IMPROVING THE NUTRITIVE VALUE OF FLOUR

VIII. LYSINE, TRYPTOPHAN, VALINE AND METHIONINE AS SUPPLEMENTS TO THE PROTEIN IN FLOUR [1]

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Many investigators have shown that the ratio of amino acids in the diet influences body functions. Berg ('55) stated that the capacity of a protein to promote growth or to offset loss of nitrogen depends on the quality and proportion of the amino acids present. This has been shown many times by Rose and co-workers ('52, '54). The importance of amino acid balance in nutrition has been ably discussed by Elvehjem and Harper ('55)

Studies on kwashiorkor by Brock and associates ('55) showed that mixtures of amino acids brought about significant improvement in cases of protein malnutrition. In parts of the world where animal proteins are not available plant proteins are used, but the mixture of plant proteins is limited and often only one type of plant protein is available. Wheat flour is used quite widely and its protein content might easily be supplemented with amino acids, provided the correct ratio is found. Westerman, Hays and Schoneweis ('57) reported that diets high in cereal content and low in meat and milk could be improved nutritionally by the addition of lysine but the optimal ratio of supplementation was not found. The data reported in this paper give information on supplement-

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ing the protein in flour with lysine, tryptophan, valine and methionine on the use of protein from legumes.

PROCEDURE

Natural foods were used in preparing 20 different diets which were patterned after those used by people with low incomes (Adelson and Blake, '50; Moser, '45). All the diets contained 3.8% sugar, 4.6% fat, 17.9% potatoes, 5.6% carrots, 8.8% green vegetables and 11.0% apples. Diets 1 to 9 had 6% milk and 42.2% enriched flour, except diet 3, with 4% milk, 36% flour, 7% meat and diets 4 and 7, which had 36.4% flour and 5.7% dried beans. Diets 10 to 20 contained 2% milk and 41.2 to 46.8% enriched flour, except diet 17 with 4% milk. Some of the 20 diets had supplements of 5.7% dried beans, which replaced a part of the flour and L-lysine·HCl, DL-valine, DL-tryptophan, DL-methionine, thiamine, riboflavin, nicotinic acid and iron. The amount of each supplement in the different diets is shown in tables 1 and 2. The method of preparation was described previously by Westerman, Linn, Templeton, and Wells ('49).

The amount of lysine added was based upon the suggestion of Block and Bolling ('44) that 3 gm of lysine per 100 gm of protein in bread would be beneficial. In some diets this amount was doubled or tripled. The quantities of tryptophan, valine and methionine added, approximated that contained in the amount of meat in diet 3. The addition of such amounts presumably would not upset the metabolic processes. Diets without meat and with only 2% milk were likely to be lacking in B vitamins as well as amino acids, therefore in some cases the amounts of B vitamins and iron in the enriched flour were doubled.

Albino rats, three weeks of age, weighing between 45 and 55 gm, were divided into groups of 10 or 12 with equal distribution as to sex and litter mates, and placed on the different diets. Another group of 12 rats was fed the stock ration of laboratory chow.[2] The animals were weighed weekly.

[2] Purina.

The food was weighed daily to determine food efficiency. At the end of the 12 weeks growth test, the animals were sacrificed and their livers taken for fat and B vitamin analysis. The thiochrome procedure of Hennessy ('41) was used for thiamine, the fluorometric method of Peterson, Brady and Shaw ('43) for riboflavin, and the microbiological methods of Strong, Feeney and Earle ('41) and Krehl, Strong and Elvehjem ('43) for pantothenic acid and nicotinic acid respectively. Fat was determined by the method of the AOAC ('50).

RESULTS AND DISCUSSION

The total average weight gains of the rats on the different diets are shown in table 1. This table also includes data on the mean food efficiency, i.e., the ratio of grams gained per gram of food eaten, and the percentage of protein in the diets. Figure 1 indicates graphically the average weight gains, while figure 2 shows the growth curves of animals on those diets with considerable differences in food composition. The influence of the dietary supplements on the B vitamins and fat content of the liver is shown in table 2.

Comparison of diets 1 to 9 containing 6% milk or milk plus meat, dried beans or lysine

Growth. The animals on the stock ration made the greatest gain during the 12 weeks test. This was significantly more ($P < 0.05$) than the animals on the other diets. Those on diets 1 and 2 made about the same gains (table 1). Animals on diets 3 and 4 had approximately the same average gains. As diet 6 had 0.42% lysine added, the rats on this diet might have been expected to have made greater gains than those on diet 5 with 0.14% lysine. However such was not the case, as the total weight gains were 214 and 213 gm respectively. A decrease to 208 gm occurred when lysine was omitted and 5.7% beans were added in diet 7 and a further decrease to 202 gm occurred with diet 8 and a still further drop to 192 gm for the animals on diet 9 (fig. 1). The differences in the

weight gains made by the rats on diets 1 to 9 were non-significant statistically.

It appears that for growth purposes a diet with 6% milk and 0.28% lysine added, as in diet 1, is equivalent to diet 3 with 4% milk and 7% meat, as the rats on these diets

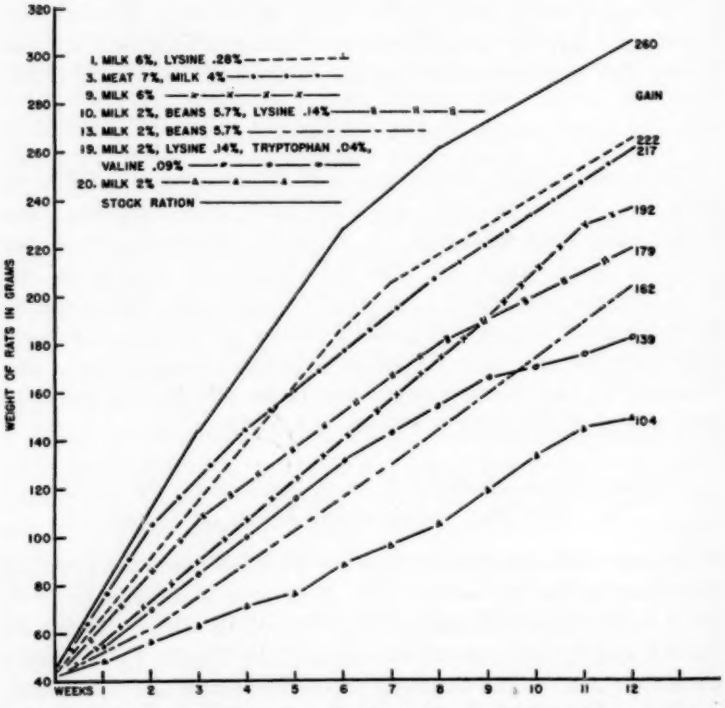

Fig. 1 Chart showing total average weight gains of rats on the different diets.

made approximately the same gains (fig. 2). However the gains of animals on these two diets were not quite statistically significant over those of the animals on diet 9 without supplements.

Food efficiency. The data on food efficiency (table 1) were not as consistent as the growth rate. Diet 1 had a

significantly greater ($P < 0.05$) food efficiency (0.227) than all the other diets except diet 5 and almost greater than diet 6, which had food efficiencies of 0.197 and 0.201 respectively. However the food efficiencies of diets 5 and 6 were significantly

TABLE 1

Mean weight gains and food efficiency of rats on the different diets

DIET NO.	SUPPLEMENT [1]	GAIN [2]	FOOD EFFICIENCY [2]	PROTEIN
	%	gm		% DRY WT.
	Diets with 6% milk, except diet 3 with 4%			
Stock		260		
1	Lysine, 0.28	222	.227	12.6
2	Lysine, 0.14; tryptophan, 0.04; valine, 0.09	220	.185	12.8
3	Milk, 4; meat, 7; eggs, 1.5	217	.157	13.1
4	Beans, 5.7; lysine, 0.14	215	.189	13.6
5	Lysine, 0.14	213	.197	11.9
6	Lysine, 0.42	214	.201	12.2
7	Beans, 5.7	208	.193	13.1
8	Eggs, 1.5	202	.159	12.4
9	No supplement	192	.181	12.2
	Diets with 2% milk, except diet 17 with 4%			
10	Beans, 5.7; lysine, 0.14	179	.145	12.3
11	Lysine, 0.42	176	.149	11.9
12	Lysine, 0.14; B vitamins, iron [3]	169	.175	11.6
13	Beans, 5.7	162	.108	12.1
14	Lysine, 0.14; tryptophan, 0.04; valine, 0.09; methionine, 0.06; B vitamins and iron	160	.191	11.5
15	Lysine, 0.14; tryptophan, 0.04; valine, 0.09; methionine, 0.06	160	.158	11.8
16	Lysine, 0.14; tryptophan, 0.04; valine, 0.09; B vitamins, iron	157	.168	11.8
17	Milk, 4; eggs, 1.5	157	.137	12.1
18	Lysine, 0.14	143	.113	11.1
19	Lysine, 0.14; tryptophan, 0.04; valine, 0.09	139	.140	11.2
20	No supplement	104	.100	11.4

[1] The amino acids were supplied as L-lysine HCl, DL-tryptophan, DL-methionine and DL-valine.

[2] Analysis of variance: Differences due to diet very highly significant ($P < 0.001$) for both weight gains and food efficiency; least significant difference for all diets: growth, 39 and food efficiency, 0.029.

[3] Diets 12, 14, and 16 had 5.2 μg/gm thiamine, 3.3 μg/gm riboflavin, 44 μg/gm nicotinic acid and 34 μg/gm iron added to the flour.

higher ($P < 0.05$) than either the 0.157 and 0.159 food efficiencies of diets 3 and 8 respectively. The higher lysine content of diets 1 and 6 (table 1) probably accounted for the greater food efficiencies of these diets.

Vitamin deposition in liver. Animals on all the diets, except diets 3 and 8 which were significantly lower, showed no significant differences in thiamine content of the livers from those on the stock ration (table 2). The riboflavin content of those on the stock ration was significantly less than those on diet 4 but significantly greater than those on diets 2, 5, 8 and 9 ($P < 0.05$). The addition of beans in diet 4 may have provided more riboflavin for deposition in the liver. Pantothenic acid content in the livers of animals on the stock ration averaged 51 μg/gm and was significantly less ($P < 0.05$) than in those on the other diets except diets 1, 2 and 3. Only animals on diet 1 showed a significantly higher ($P < 0.05$) amount of nicotinic acid in the livers than those on the stock ration. Animals on diet 1 varied significantly from those on the stock ration in nicotinic acid while those on diet 2 varied in riboflavin. Those on diet 3 never varied significantly in vitamin content from those on the stock ration, while a significant variation occurred in only one vitamin, usually riboflavin or pantothenic acid, for the rats on diets 6 and 7.

Fat content of liver. A fat content of 3.5% and 3.2% in livers of rats on diets 4, 6 and 7 was significantly higher than occurred in those of the stock ration with 2.6% (table 2). As these differences were actually quite small, it is doubtful they could be considered abnormal since the amount of fat is quite low. Therefore the ratio of amino acids added to the flour in the different diets did not result in deposition of excess fat in the liver.

Comparison of diets 10 to 20 containing 2% milk or milk plus dried beans, amino acids and B vitamins

Growth. Diets 10 to 20 contained 2% milk except diet 17 which had 4%. The growth rate of rats on these diets was

considerably less than that of those on diets 1 to 9 (table 1). All diets with 2% milk plus supplements, diets 10 to 18, produced significantly better average growth gains in the animals than diet 20, with 2% milk and no supplements

TABLE 2

Influence of dietary supplements on B vitamin and fat content of liver

(All values as means. Vitamin data on a dry, fat-free basis)

DIET NO.	SUPPLEMENT	THIAMINE	RIBOFLAVIN	PANTOTHENIC ACID	NICOTINIC ACID	FAT
	%	μg/gm	μg/gm	μg/gm	μg/gm	%
	Diets with 6% milk, except diet 3 with 4%					
Stock		29	72	51	468	2.6
1	Lysine, 0.28	26	69	77	567	2.9
2	Lysine, 0.14; tryptophan, 0.04; valine, 0.09	28	57	78	468	3.0
3	Milk, 4; meat, 7; eggs, 1.5	24	82	61	470	2.9
4	Beans, 5.7; lysine, 0.14	27	92	158	506	3.5
5	Lysine, 0.14	30	48	81	452	2.7
6	Lysine, 0.42	29	79	123	438	3.5
7	Beans, 5.7	30	69	119	503	3.2
8	Eggs, 1.5	24	46	83	454	2.5
9	No supplement	28	55	89	466	2.5
	Diets with 2% milk, except diet 17 with 4%					
10	Beans, 5.7; lysine, 0.14	38	91	136	554	3.5
11	Lysine, 0.42	34	98	108	553	3.5
12	Lysine, 0.14; B vitamins; iron	44	45	104	492	2.5
13	Beans, 5.7	35	69	143	441	3.3
14	Lysine, 0.14; tryptophan, 0.04; valine, 0.09; methionine, 0.06; B vitamins and iron	28	55	58	506	2.6
15	Lysine, 0.14; tryptophan, 0.04; valine, 0.09; methionine, 0.06	32	56	129	524	2.2
16	Lysine, 0.14; tryptophan, 0.94; valine, 0.09; B vitamins; iron	45	54	52	520	2.6
17	Milk, 4; eggs, 1.5	24	47	85	411	2.6
18	Lysine, 0.14	36	65	71	499	2.5
19	Lysine, 0.14; tryptophan, 0.04; valine, 0.09	27	54	77	508	2.3
20	No supplement	34	48	128	429	3.9

Analysis of variance: Differences due to diet, very highly significant for all the vitamins and for fat ($P < 0.001$). Least significant difference for all diets: thiamine 5, riboflavin 12, pantothenic acid 29, nicotinic acid 54, fat 0.5.

($P < 0.05$). The effect of the dried beans, in diet 10, is shown by an average weight gain of 179 gm compared with 143 gm for those on diet 18, although both diets contained 0.14% lysine. The difference was very nearly significant ($P < 0.05$). Differences of 40 gm and 75 gm respectively, between diet 10 and diets 19 and 20 were significant ($P < 0.05$) indicating that the amounts of lysine, tryptophan and valine added were not sufficient to replace the beans in diet 10 for

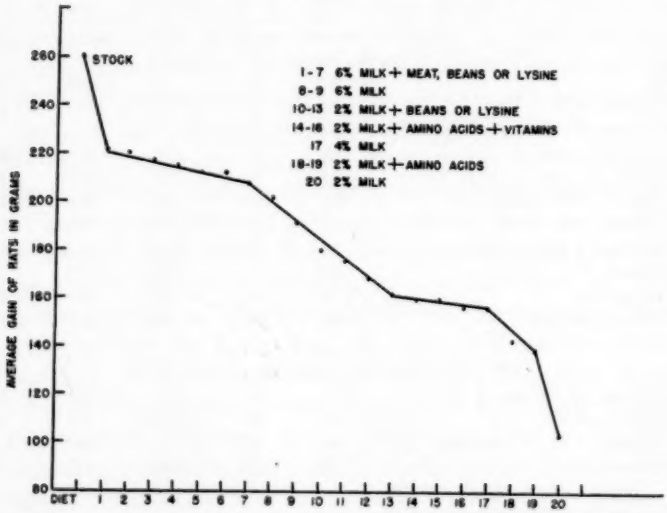

Fig. 2 Curves showing growth of animals on some of the diets.

growth purposes. However, the addition of lysine in diet 18 (fig. 1) increased the growth rate above those on diet 20 with no supplement ($P < 0.05$).

When the milk was increased to 4%, diet 17, the gain was greater than that of the animals on diets 18, 19, 20. However the gains made by animals on diet 16, which was supplemented with three amino acids, B vitamins and iron, were the same as those on diet 17, indicating that the supplements could replace part of the milk. The addition of methionine with the other

supplements, diet 14, or with the vitamin and iron supplements omitted, diet 15, produced the same weight gains, 160 gm. Evidently this amino acid was not the limiting factor.

The average weight gains of animals on diet 13 which contained beans, was less than those on diet 10 with beans and lysine (fig. 2). Those on diet 11 with 0.42% lysine added and those on diet 12 with 0.14% added lysine plus B vitamins and iron made better gains than when beans alone were present, diet 13. The differences were non-significant statistically. The gain of the rats on diet 11 was very nearly significant over the gain of those on diet 19 with 0.14% lysine and with tryptophan and valine added. Evidently the additional lysine was beneficial in promoting growth when the diet contained only 2% milk. The 0.14% lysine in diet 10 did not produce significant gains over those on diet 13 without lysine. Both diets contained beans. Gains made by animals on diets 10 and 13 were significantly greater ($P < 0.05$) than for those on diet 20 with no supplements (fig. 2). Rats receiving additional lysine, tryptophan and valine in diet 19 showed very nearly significant gains over those on diet 20. These data would seem to indicate some benefits of growth rate which could be attributed to the supplements in these diets.

Food efficiency. All the diets with 2% milk and supplements of amino acids alone or with B vitamins produced higher food efficiencies in the rats than did diet 20 with 2% milk and no supplement (table 1). Animals on diets 12, 14 and 16, which had B vitamins and iron added, as well as amino acids, produced the highest food efficiencies in this group, 0.175, 0.191 and 0.168 respectively, thus indicating some beneficial effects of the added vitamins when the milk content of the diet is low. When the milk content was increased to 6% and 0.28% lysine was added, as in diet 1, the food efficiency of 0.227 was significantly greater (table 2) than that of diets 10 to 20 ($P < 0.05$). A food efficiency of 0.140 for diet 19 was very nearly significantly more than the 0.113 of diet 18

($P < 0.05$). Evidently the addition of tryptophan and valine in diet 19 was of value in this regard.

The total protein content of the 20 diets varied from 13.6% to 11.1% (table 1). Those with lower milk content had the lower protein content which may in part account for the slower growth and lower food efficiencies of animals on these diets.

Vitamin deposition in liver. Diet differences were highly significant for each of the 4 vitamins studied ($P < 0.001$). Animals on diets 10, 12 and 16 had the highest thiamine content in the livers (table 2) significantly higher ($P < 0.05$) than the animals on the stock ration. Diets 10 and 11 allowed a significantly higher amount of riboflavin to be deposited than did the stock ration. However those on diets 12, 14 to 20 had significantly less than the stock ($P < 0.05$). Animals on diets 10 to 20, with the exception of those on diets 14, 18 and 19, had significantly more pantothenic acid ($P < 0.05$) in the livers than those on the stock ration. While animals on diets 10, 11 and 15 had a significantly higher amount of nicotinic acid than the stock.

Fat content of liver. The rats on diets 10, 11, 13 and 20 averaged 3.3 to 3.9% of fat in the livers, which was significantly higher than the 2.6% of those on the stock ration. It seems unlikely that such small differences could be considered abnormal. The amino acid supplements did not upset the normal amino acid metabolism as far as deposition of fat in the livers was concerned.

SUMMARY

Data have been presented relating to the addition of lysine, tryptophan, valine and methionine to diets high in cereal containing 2% and 6% milk. All diets with 2% milk and added supplements produced significantly better growth gains and food efficiencies in the rats than the diet with 2% milk alone. The addition of 5.7% beans with 0.14% lysine or 0.42% lysine without beans promoted the best growth rates on diets with 2% milk. However, the addition of lysine did not increase the growth rate or food efficiency, unless vitamins

were added, up to that of the animals with 6% milk in the diet.

The addition of lysine to diets with 6% milk produced no statistically significant weight gains over those of animals on diets without lysine. The amounts of tryptophan, valine and methionine added along with the lysine in some diets was not sufficient to replace beans for growth purposes.

Animals with 2% milk, 5.7% beans and 0.14% lysine in the diet stored significantly more of the 4 B vitamins and fat in the livers than did the stock animals. Those on diets with 6% milk, 5.7% beans and 0.14% lysine and those with 2% milk with 0.42% lysine stored more riboflavin, pantothenic acid and fat than those on the stock ration.

Under the conditions of these experiments the addition of lysine up to 0.42% of the diet did not produce an imbalance in the amino acid ratio, or upset the normal metabolic processes.

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THE EFFECT OF INCREASED DIETARY FAT UPON THE PROTEIN REQUIREMENT OF THE GROWING DOG[1]

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The work of Forbes et al. ('38) and Combs and Romoser ('55) suggests that the dietary level of protein should be based upon the available energy content of the diet. Sunde ('56) obtained increased growth of chicks with added increments of fat to a diet which contained 28% protein. Campbell and Phillips ('53) found that added increments of fat to a diet for growing dogs inhibited the growth rate when the protein level was 19.7% and that the normal growth rate was resumed when a supplement of 0.3% methionine was added. An effort has been made to extend these observations and determine the protein requirement of the growing dog when a good quality mixed protein was present in a high-fat diet.

EXPERIMENTAL

In the first of two experiments, 4 lots of weanling pups were fed diets containing 20% fat and 16, 20.5, 25 and 29.5% protein. In the second experiment, 4 more lots were fed diets which contained 30% fat and 19.9, 24.4, 28.9 and 33.4% protein. There were 10 to 11 animals in each lot. The complete diets are presented in table 1.

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The pups were allotted by litter mate distribution, and represented 11 litters per experiment. They were housed indoors upon expanded-metal-bottom cages with two to three pups per cage. Proper precautions were taken to avoid parasites, distemper and infectious canine hepatitis especially with young pups obtained outside our kennel. Each group was composed of beagle, shepherd and shepherd-collie pups. They were allotted to their respective groups at approximately 6

TABLE 1

Percentage composition of diets

CONSTITUENT	EXPERIMENT 1	EXPERIMENT 2
	%	%
Corn, yellow, no. 2 dent, ground	30	14
Soybean meal	15	14
Skim milk, dried	10	10
Alfalfa leaf meal	5	5
Brewers' yeast, dried	3	5
NaCl (iodized)	1	1
$CaHPO_4 \cdot 2H_2O$	2	2
Lard [1]	19	29
Casein, crude	0–15 [2]	6–21 [3]
Sucrose	15–0 [2]	15–0 [3]
Yeast, irradiated [4]	0.01	0.01

[1] Oscar Mayer prime steam lard.
[2] Diets 1, 2, 3 and 4 contained 0, 5, 10, 15% casein and 15, 10, 5, 0% sucrose.
[3] Diets 1, 2, 3 and 4 contained 6, 11, 16, 21% casein and 15, 10, 5, 0% sucrose.
[4] Contained 900 USP units of vitamin D_2 per gram.

weeks of age and were conditioned to the experimental routine for the first week during which all lots were fed the basal ration only. Thereafter the experimental regimen was followed for 10 weeks. Food and water were given ad libitum.

RESULTS

Data on growth, food efficiency, and the Kjeldahl analyses ($N \times 6.25$) are presented in table 2.

In the 20% fat experiment the rate of growth increased progressively as the dietary protein was increased up to 25.0%. Beyond this level the dogs grew no faster than those

fed the 25.0% protein. When the dietary fat content was raised to 30%, the rate of growth increased as the dietary protein increased up to 28.9%. The addition of casein which raised the total protein to 33.3% failed to stimulate growth further.

TABLE 2

Growth of dogs as affected by the ratio of fat to protein in the diet

CATEGORY OF INTEREST	LOT			
	1	2	3	4
Experiment 1 — 20% fat				
Per cent dietary protein	16.0	20.5	25.0	29.5
Average initial weight [1]	1.81	1.88	1.86	1.87
Average final weight	6.96	7.95	8.57	8.65
Average gain	5.15	6.07	6.71	6.78
Comparative gain [2]	76	90	99	100
Food efficiency [3]	3.2	2.6	2.1	2.2
Food efficiency [4]	14.40	11.70	9.45	9.90
Experiment 2 — 30% fat				
Per cent dietary protein	19.9	24.4	28.9	33.4
Average initial weight [1]	2.13	2.09	2.15	2.16
Average final weight	6.93	7.58	8.53	8.41
Average gain	4.80	5.49	6.38	6.25
Comparative gain [2]	75	86	100	98
Food efficiency [3]	2.4	2.1	1.8	1.8
Food efficiency [4]	12.29	10.75	9.22	9.22

[1] All weights expressed in kilograms.
[2] The average gain in each lot was expressed as a percentage of the heaviest lot.
[3] Kilograms of feed per kilogram gain in body weight.
[4] Calories $\times 10^{-3}$ per kilogram gain in body weight.

More rapid gains in body weight were obtained when the protein-fat or protein-calorie balance was optimum. In experiment 1 maximum growth and the greatest food efficiency were obtained when the diet contained 25% protein and 20% fat, whereas these maxima were approached in experiment 2 when the ratio of protein to fat was 28.9 : 30. The significance of these figures is not presently evident. There was a uniformly higher food efficiency in the pups fed the 30% fat

diets over those fed the 20% fat diets. It was also observed that the larger pups were more sensitive indicators of optimal dietary conditions in this respect than were the pups of the smaller breeds.

All animals remained free of infectious diseases throughout these experiments and were healthy, normal pups. It was noticeable, however, that a few individual pups in lot 1, experiment 1 and those in lot 1, experiment 2 exhibited signs of low protein intake, namely, poor haircoat and "lack of thrift," equivalent to a sub-clinical protein deficiency syndrome.

DISCUSSION

Optimum growth and optimum feed efficiency would seem by these studies to be closely related to a rather sensitive balance between the amount of protein and the caloric content of the diet. Gessert and Phillips ('56) achieved the best growth rate in young dogs with 17.2% of dietary protein when approximately 7.5% fat was present; the ration has been estimated to contain 3.94 Cal./gm with a food efficiency of 3.85 kg/kg of gain in weight. The caloric contents of the rations used in experiment 1 and 2 were estimated at 4.50 and 5.12 Cal./gm, respectively, with maximum food efficiencies of 2.1 and 1.8 kg of feed/kg of body weight gain. These data indicate a more effective use of protein or fat or both in the presence of a proper balance between the two. The point of maximum growth of weanling puppies in these experiments was attained when the ratio of calories to grams of protein was 18 : 1.

It should be pointed out that only young growing pups were used in these studies and the period under test covered 10 weeks which is the most critical time in the growth cycle of the dog. Whether the efficiency of the gastrointestinal tract is greater during this period, or whether a smaller food intake makes for less digestive congestion and hence more effective and complete digestion and assimilation are matters of conjecture at this time.

The results obtained in these experiments fit into the pattern of observations made by other workers. Combs and Romoser ('55), Sunde ('56), Leong et al. ('55), Donaldson et al. ('56), among others have demonstrated that the percentage of dietary protein required by the chicken is increased by added increments of energy. Greenberg and coworkers ('51) showed that the highest food efficiency was obtained with rats which were fed a diet containing 30% fat in comparison with those fed a similar diet but low in fat and supplemented with the essential fatty acids. In a study of the comparative nutritive value of lard and hydrogenated cottonseed oil Hoagland and Snider ('41) found that the feeding of either fat stimulated maximum growth in rats. Lard was superior to the cottonseed oil at dietary levels of fat higher than 5%. That a difference in the nutritive values of different oils and fats exists is supported by the work of Thomasson and Boldingh ('55). Thomasson ('56) as well as Steenbock et al., ('36) were able to demonstrate a difference in the absorption rates for different oils and fats. In the present studies the factor of absorption was of little consequence except possibly as the dietary fat increments were increased, and this seems unlikely in view of the fact that the high-fat diets resulted in greater growth responses in the test animals.

The consensus of opinion is that animals of a given species eat to satisfy their calorie requirements, hence, a reduction in food intake with the higher fat diets was not surprising. The growth response to high-fat diets in these experiments, in contrast to those obtained by Gessert and Phillips ('56), suggests that the major suboptimal dietary component of the latter diet for the weanling pup was one of calories. To match optimum calorie intake requirement it was necessary to increase the protein percentage of the diet. From results of the earlier work of Campbell and Phillips ('53) amino acid components of the protein may properly supplement the high calorie diets. Such test studies are currently in progress.

SUMMARY

The effect of a high-fat, or high-calorie diet upon the percentage protein requirements of the weanling pup has been investigated. The data show that, under ad libitum feeding, increased increments of dietary fat in the ration of the weanling dog increased the present protein requirement as measured by rate of growth and by food efficiency.

The diet which contained 20% fat needed 25.0% protein to obtain a maximum growth response in the weanling pup in these experiments. Further increases in protein were needed to obtain a maximum growth and food efficiency rate when the diet contained 30% fat. The food efficiency for the weanling pup was distinctly superior with the 30% fat diet over that with the diet which contained 20% fat.

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THE FOOD INTAKE AND NITROGEN RETENTION OF WEANLING RATS FED PROTEIN-FREE RATIONS [1,2]

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The classical studies of W. C. Rose at Illinois have enabled the formulation of growth-promoting rations devoid of protein but containing the indispensable amino acids. Rose et al. ('49) fed weanling rats a ration containing the 10 indispensable amino acids in the smallest quantities which would permit maximum growth. Supplementing this ration with certain nitrogen-containing compounds resulted in a marked acceleration in growth; diammonium citrate was the most active supplement used. Womack et al. ('53) were unable to improve negative nitrogen balances occurring in adult rats, fed low levels of the indispensable amino acids, by supplementing the diets with levels of 0.73 and 2.5% diammonium citrate. Finlayson and Baumann ('56) reported that the addition of 10% diammonium citrate to a purified rat diet resulted in a growth depression of 27% over a 5-week period when the rats were fed ad libitum.

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In a report of an earlier experiment, in which a casein-lactalbumin mixture supplemented with amino acids was used as the nitrogen source, Sibbald et al. ('57a) noted a high, inverse relationship between the weight of food consumed by weanling rats and the A.D.E.[3] content of the food.

TABLE 1

Composition of nitrogen source "B"

	NITROGEN SOURCE "B"		WT. OF ACTIVE AMINO ACIDS IN 130.5 GM NITROGEN SOURCE "A" [1]
	Wt. of nutrients used	Wt. of active amino acids	
	gm	*gm*	*gm*
L-Arginine HCl	4.9	4.1	4.1
L-Histidine HCl	5.3	3.9	3.9
DL-Isoleucine	15.0	7.5	7.5
L-Lysine HCl	11.8	9.5	9.5
DL-Methionine	5.8	5.8	5.8
DL-Phenylalanine	6.7	6.7	5.7 [2]
DL-Threonine	11.4	5.7	5.7
DL-Valine	14.2	7.1	7.1
L-Leucine	11.0	11.0	11.0
DL-Tryptophan	1.9	1.9	1.9
Ammonium citrate	37.9	..	..
Corn starch	4.6	..	..
Total	130.5	..	..
% Nitrogen	12.57	..	12.57

[1] Block, R. J., and D. Bolling 1951 The Amino Acid Composition of Proteins and Foods. Charles C Thomas, Publ., Springfield, Illinois.

[2] Although this value is low there is sufficient tyrosine present in casein and lactalbumin to supplement the phenylalanine. Womack and Rose ('46) have shown that tyrosine can reduce the phenylalanine requirement of the rat from 0.9 to 0.45% of the total diet.

It was also observed that a curvilinear relationship existed between the percentage A.D.N. retained and the A.D.E. per gram A.D.N. in the diet.

The experiment reported herein was designed to study the influence of A.D.E. on the food consumption and nitrogen

[3] Throughout this report the following abbreviations are employed: A.D.E. apparent digestible energy and A.D.N. apparent digestible nitrogen.

retention of the weanling rat when the nitrogen source in the rations consisted of a mixture of indispensable amino acids and diammonium citrate.

The influence of the dry density and gross nitrogen level of the ration on food consumption was also investigated.

METHODS

The composition of nitrogen source "B" which was used in this experiment is outlined in table 1. In the formulation of this mixture the indispensable amino acids were included in the same quantities as they were calculated to occur in nitrogen source "A", a casein-lactalbumin mixture supplemented with amino acids (Sibbald et al., '56, '57a). Diammonium citrate and cornstarch were added to the amino acid mixture in such proportions that the nitrogen content of source "B" was the same as that of nitrogen source "A".

In the formulation of the experimental rations 4 levels of Alphacel,[4] a non-nutritive cellulose, were combined with 4 levels of nitrogen; additions of Alphacel and the nitrogen source being made at the expense of the sucrose portion of the ration. As previous data (Sibbald et al., '56) indicated Alphacel to be indigestible, the rations formulated were anticipated to exhibit 4 levels of A.D.E. which in combination with the 4 levels of nitrogen source "B" would yield varying levels of A.D.E. relative to A.D.N. The composition of the experimental rations is listed in table 2.

Three male and three female weanling rats of the Sprague-Dawley strain were alloted to each of the 16 rations listed in table 2. Following a 7-day ration acclimatization period the rats were placed in individual metabolism cages for an experimental period of 7 days. The experimental period was limited to one week as the rats on certain rations were growing rapidly and variations in weight, resulting from the feeding

[4]Alphacel, "non-nutritive cellulose." Nutritional Biochemicals Corporation, Cleveland, Ohio.

of rations differing in nutritive value, would have been magnified if a longer period had been employed.

Feces and urine were collected, prepared and analyzed by the methods outlined by Sibbald et al. ('57a). A.D.E. consumption was determined by subtracting total fecal energy

TABLE 2

Rations[1] fed during acclimatization and metabolism periods

RATION	ALPHACEL[2]	NITROGEN SOURCE "B"[3]	SUCROSE	ANALYSIS		DENSITY
				Gross energy	Gross nitrogen	
	%	%	%	*Cal./100 gm*	*mg/100 gm*	*gm/ml*
1a	10	10.2	69.8	409	1351	0.518
1b	10	15.2	64.8	409	1903	0.532
1c	10	20.3	59.7	407	2507	0.460
1d	10	25.4	54.6	413	3211	0.433
2a	20	10.2	59.8	409	1330	0.402
2b	20	15.2	54.8	405	1955	0.417
2c	20	20.3	49.7	415	2583	0.398
2d	20	25.4	44.6	419	3112	0.353
3a	30	10.2	49.8	410	1350	0.333
3b	30	15.2	44.8	413	1923	0.328
3c	30	20.3	39.7	422	2522	0.338
3d	30	25.4	34.6	424	3211	0.307
4a	40	10.2	39.8	408	1338	0.287
4b	40	15.2	34.8	418	1973	0.277
4c	40	20.3	29.7	420	2529	0.268
4d	40	25.4	24.6	426	3168	0.253

[1] Each ration contained 5% Mazola oil, 4% salts (Jelinek et al., '52) and 1% vitamin mix (Sibbald et al., '56).

[2] Alphacel, "non-nutritive cellulose." Nutritional Biochemicals Corporation, Cleveland, Ohio.

[3] Nitrogen Source "B" composition listed in table 1.

from the gross energy intake; A.D.N. consumption was determined in a similar manner. The A.D.E. and A.D.N. content of the rations was calculated from the intake of these factors and the food consumption. Nitrogen retention was determined by subtracting gross urinary nitrogen excretion from the A.D.N. consumption.

Food density was measured by determining the weight of dry food required to fill a standard imperial pint measure. The food was poured into the cylinder from a height of three inches and the excess removed by a two inch diameter glass roller. The dry food density was computed as grams per milliliter.

Data were collected separately for individual rats and all subsequent computations and statistical analyses are based on individual and not group average data. Since most of the variables studied were influenced by body weight it was necessary to remove any variation associated with this factor prior to determining the magnitude of the various relationships under consideration. This was accomplished by expressing the variables on a 100 gm body weight basis. The body weight referred to was the average of the initial and final body weights of the rats during the experimental period.

In some of the computations relative to food consumption the reciprocals of the A.D.E. and gross nitrogen content of the diets were used in preference to the natural values. Plotting the natural values gave a curvilinear relationship, symmetrical about the main axes, which indicates that a proportional relationship probably existed between the variables. The reciprocals were thus found to give a truer picture of the biological association involved and are therefore used throughout this report. A more detailed discussion of the reasons for this procedure are presented by Sibbald et al. ('57a).

RESULTS

The gross energy and nitrogen content of each of the experimental rations are listed in table 2. Also presented in this table are the dry density values of the diets which it will be noted decrease as the Alphacel content of the ration increases.

Food consumption. The principal mean values, relative to food consumption, are presented in table 3. Examination of these results indicates that as the level of Alphacel in the

ration was increased (10, 20, 30 and 40%) the A.D.E. content decreased and the food consumption per 100 gm body weight increased. The A.D.E. consumption per 100 gm body weight remained relatively constant between ration groups indicating that the decreasing A.D.E. levels of the rations were compensated for by increased food consumption. The increase

TABLE 3

Mean values for data relating to the effect of apparent digestible energy (A.D.E.) and gross nitrogen on food consumption

RATION	BODY WT. OF RATS	FOOD CONSUMPTION/ 100 GM BODY WT.		GROSS NITROGEN CONSUMPTION/ 100 GM BODY WT.	A.D.E. PER		
					100 gm food	100 ml food	100 gm body wt.
	gm	*gm*	*ml*	*mg*	*Cal.*	*Cal.*	*Cal.*
1a	50	68	131	919	368	191	250
1b	52	79	148	1503	368	197	291
1c	58	76	165	1905	365	168	278
1d	53	76	176	2440	373	161	283
2a	48	79	197	1051	333	134	264
2b	56	83	199	1623	328	138	274
2c	61	78	196	2015	334	133	260
2d	54	82	232	2552	342	122	282
3a	51	85	255	1148	296	98	251
3b	54	90	274	1731	296	96	266
3c	54	87	258	2194	297	100	257
3d	50	89	290	2858	308	94	273
4a	51	100	348	1325	257	75	258
4b	53	107	386	2111	270	75	289
4c	54	106	395	2681	266	71	281
4d	47	98	387	3105	266	70	272

in food consumption resulted in an increase in gross nitrogen intake.

An analysis of variance indicated a highly significant ($P < 0.01$) difference in the weight of food consumed per 100 gm body weight between rations varying in Alphacel levels. No such difference was found when the food consumption between nitrogen levels was compared. The interaction between Alphacel and nitrogen levels was not significant.

These results indicate that food consumption was neither stimulated nor depressed by increasing the level of protein-free, diammonium citrate-containing, nitrogen source "B" in the rations. A further test of the influence of the gross nitrogen level of the ration on food consumption was obtained from a correlation between the food consumption (gm/100 gm body weight) and the reciprocal of the gross nitrogen per 100 gm of food. The resulting r value of —0.091 at 94 D.F. was not significant indicating that changing levels of nitrogen source "B", and therefore diammonium citrate, did not influence food consumption. Diammonium citrate did not therefore depress or stimulate food intake unless it had already expressed itself to a maximum at the lowest level at which it was included in the experimental diets.

The food intake of the weanling rats used in this experiment varied inversely as the A.D.E. content of the rations. A correlation between the weight of food consumed per 100 gm body weight and the reciprocal of the A.D.E. per 100 gm of food yielded a highly significant r value of 0.763 at 94 D.F., while a similar correlation between the volume of food consumed and the reciprocal of the A.D.E. per 100 ml of food yielded the significantly higher ($P < 0.01$) r value of 0.964 at 94 D.F. This improved correlation indicates that in the rations used in this experiment, volume of food consumed more closely parallels the energy content of the food than does the weight of food consumed.

The data of tables 2 and 3 indicate that as the level of Alphacel in the rations increased the A.D.E. content of the food and the dry density of the diets, expressed as grams per milliliter, decreased. This strong association between the A.D.E. content of the food and the dry density complicates interpretation of the data relative to the influence of energy on food consumption because of the possibility that bulk (as measured by dry density) might itself influence food intake. Within the design of the present experiment it is probably not possible to completely resolve this problem; however, an

analysis of variance showed that there was no difference in the A.D.E. consumption of rats between density levels. This, together with the aforementioned observation that A.D.E. consumption per 100 gm body weight of the rats remained relatively constant over all rations, would lend support to

TABLE 4

Mean data relating to the influence of apparent digestible energy (A.D.E.) on nitrogen retention

RATION	GROSS NITROGEN		A.D.N.			A.D.E./GM A.D.N. IN FOOD
	Per 100 gm food	Digested	Per 100 gm food	Per 100 gm body wt.	Retained	
	mg	%	*mg*	*mg*	%	*Cal.*
1a	1351	92	1229	836	58	298
1b	1903	92	1745	1378	59	211
1c	2507	91	2277	1730	51	161
1d	3211	92	2974	2260	51	125
Mean		*92*				
2a	1330	85	1125	889	66	298
2b	1955	87	1699	1410	61	193
2c	2583	89	2298	1792	54	146
2d	3112	92	2858	2344	44	120
Mean		*88*				
3a	1350	81	1094	930	68	272
3b	1923	80	1531	1378	65	196
3c	2522	85	2143	1864	46	139
3d	3211	90	2892	2574	42	106
Mean		*84*				
4a	1338	81	1078	1078	60	239
4b	1973	84	1663	1779	52	163
4c	2529	83	2096	2222	48	128
4d	3168	86	2716	2662	34	98
Mean		*84*				

the hypothesis that food consumption was regulated basically by the energy content of the ration and that bulk was coincidental. In order to completely validate this hypothesis it would be necessary to formulate rations in which variations in the energy content and the density were not so closely associated.

Nitrogen retention. The principal mean values relative to nitrogen retention, obtained in this experiment, are presented in table 4. Increasing levels of Alphacel tended to decrease the digestibility of the gross nitrogen of the food though no difference was observed between Alphacel levels of 30 and 40%. The consumption of A.D.N. per 100 gm body weight increased as the A.D.E. per gram A.D.N. of the food decreased since the variation in food consumption was largely associated with the A.D.E. content of the rations. The percentage A.D.N. retained did not appear to be influenced by the nitrogen (and therefore diammonium citrate) intake, but rather by the A.D.E.: A.D.N. ratio of the food.

Statistical analysis following the methods outlined by Snedecor ('46) yielded the following information. A correlation between the percentage A.D.N. retained and the A.D.E. per gram A.D.N. in the food yielded the highly significant r value of 0.739 at 94 D.F. A test for curvilinearity of regression demonstrated a significantly ($P < 0.01$) improved relationship, a highly significant R value of 0.807 at 93 D.F. indicated an association of 65% between the variables. The quadratic equation obtained for curvilinear regression was:

$$Y = -2.8 + 0.507X - 0.000959X^2$$

where Y is the percentage A.D.N. retained and X the A.D.E. per gram A.D.N. in the food. This curve (which is graphed in the succeeding report Sibbald et al., '57b) gives a maximum for Y of 65.2% with X at 264 Cal. A.D.E. per gram A.D.N. The original standard deviation in the percentage A.D.N. retained was 11.1% and the standard error of estimate for deviations from curvilinear regression ($s_{y \cdot x}$), which indicates the variation remaining after removing that associated with the A.D.E.: A.D.N. ratio of the food, was 6.6%.

DISCUSSION

Food consumption. The results of this experiment indicate that the nitrogen level of the rations, and hence the diammonium citrate content, did not exert any significant influence upon

food intake. It was noted that 58% of the variance in the weight of food consumed was inversely associated with the A.D.E. content of the food. Although a greater association (93%) was found between the volume of food consumed and the reciprocal of the A.D.E. per 100 ml of food this was probably of little biological significance due to the very high association between the A.D.E. content of the food and the density. It was explained in the section entitled Results that any relationship between food intake and density was probably coincidental but that it was beyond the scope of the present experiment to validate this hypothesis.

Nitrogen retention. Analysis of the nitrogen retention data indicated a curvilinear relationship between the percentage A.D.N. retained and the A.D.E. per gram A.D.N. An estimated maximum of 65.2% of the A.D.N. was retained when the A.D.E. per gram A.D.N. was 264 Cal. Using a nitrogen source based on a casein-lactalbumin mixture supplemented with amino acids, Sibbald et al. ('57a), using a similar method, derived an estimated maximum of 89.3% when the A.D.E. per gram A.D.N. was 277 Cal. The similarity of the two optimal levels of A.D.E. per gram A.D.N. (264 and 277 Cal.) raises the question as to whether the optimal ratio of A.D.E. per gram A.D.N. for all nitrogen sources falls in close proximity to these values. A preliminary investigation into this problem is presented in the succeeding paper (Sibbald et al., '57b).

Several interesting points arise from the nitrogen retention data of this report and that of Sibbald et al. ('57a). In the determination of the biological values of proteins it is generally considered that low levels of protein yield the most accurate results. It is widely accepted that as the level of protein in the ration increases the biological value decreases (Barnes et al., '46). However, if, as has been shown, the relationship between nitrogen retention and the A.D.E. per gram A.D.N. in the food is curvilinear, then it would appear that it is not so much the level of protein in the food which is important but rather the ratio of A.D.E. to A.D.N.

The percentage A.D.N. retained corresponds to the biological value for growth and differs from the biological values obtained by the Thomas-Mitchell method inasmuch as the latter makes correction for the metabolic fecal and the endogenous urinary nitrogen excretion. Biological values are frequently expressed as percentages of the biological value of a protein known to be of high quality. A comparison of this nature in which the percentage A.D.N. retained by rats

TABLE 5

The influence of the ratio of the apparent digestible energy (A.D.E.) to the apparent digestible nitrogen (A.D.N.) on the biological value for growth [1]

NITROGEN SOURCE "B"	NITROGEN SOURCE "A"						
A.D.E. per gm A.D.N.	100	150	200	250	264	277 [2]	300
Cal.							
100	*74.9*	55.0	46.6	43.3	43.0	42.9	43.2
150	101.0	*74.1*	62.9	58.4	58.0	57.8	58.3
200	117.8	86.4	*73.3*	68.1	67.6	67.4	67.9
250	125.1	91.8	77.9	*72.4*	71.8	71.6	72.2
264 [2]	125.5	92.1	78.2	72.6	*72.0*	71.9	72.4
277	125.2	91.9	78.0	72.4	71.9	*71.7*	72.2
300	122.2	90.4	76.7	71.2	70.7	70.5	*71.0*

[1] The percentage apparent digestible nitrogen (A.D.N.) retained of Nitrogen Source "B" expressed as a percentage of the percentage A.D.N. retained of Nitrogen Source "A" at varying ratios of A.D.E.:A.D.N. Data relative to Nitrogen Source "A" from the report of Sibbald et al. ('57a).

[2] Level of A.D.E. per gram A.D.N. which results in a maximum percentage of A.D.N. being retained.

consuming nitrogen source "B" is expressed as a percentage of the percentage A.D.N. retained by rats consuming a casein-lactalbumin mixture supplemented with amino acids (Nitrogen source "A", Sibbald et al., '57a), at varying levels of A.D.E. per gram A.D.N. in the food, is given in table 5.

The data of table 5 indicate that at equal levels of A.D.E. per gram A.D.N. the biological value for growth of nitrogen source "B" expressed as a percentage of that for nitrogen source "A" (figures in italics) is not constant but changes with the A.D.E.: A.D.N. ratio of the food. When the level of

A.D.E. per gram A.D.N. for the two nitrogen sources differs the percentage biological value for growth of nitrogen source "B" exhibits even greater variation except in the range 250 to 300 Cal. per gram A.D.N. which occupies the plateaux of the two curves. It would therefore appear logical to determine the biological values of proteins, or nitrogen sources, either at some constant level of A.D.E. per gram A.D.N. which ensures that the percentage A.D.N. retained is close to a maximum (i.e. on the plateau of the curve) or at a level of A.D.E. per gram A.D.N. which results in a maximum percentage of A.D.N. being retained. Table 5 demonstrates quite clearly that as one moves away from the plateaux of the curves greater variation is encountered.

SUMMARY

Variations in the food consumption of weanling rats, fed rations containing a mixture of indispensable amino acids and diammonium citrate, were largely associated with the apparent digestible energy (A.D.E.) content of the diets. The influence of bulk (as measured by dry density) on food intake could not be entirely resolved but there was some indication that the influence of dry density on food consumption resulted from its high association, in these experiments, with the A.D.E. content of the food. The gross nitrogen level of the rations, and hence the diammonium citrate content, did not exert any significant influence on food consumption.

The ratio of A.D.E. to apparent digestible nitrogen (A.D.N.) was associated with 65% of the variance in the percentage A.D.N. retained. This relationship was curvilinear yielding an estimated maximum for the A.D.N. retained of 65.2% with the A.D.E. per gram A.D.N. at 264 Cal.

The importance of A.D.E. in determining the biological values of proteins or nitrogen sources was discussed. It would seem necessary to determine biological values when the ratio A.D.E.: A.D.N. of the food results in the percentage of A.D.N. retained being situated on the plateau of the curve.

Perhaps the most accurate data would be obtained when the A.D.E. per gram A.D.N. results in a maximum percentage of A.D.N. being retained.

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THE INFLUENCE OF THE NITROGEN SOURCE ON THE FOOD INTAKE AND NITROGEN RETENTION OF WEANLING RATS[1]

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In an interesting review of some of the factors regulating energy intake and body weight, Mayer ('55) indicated that both rabbits and rats regulate their food intake according to their energy needs. Janowitz and Hollander ('55) reported that when 50 or 100% of the calculated caloric requirements of dogs was introduced into the stomach per fistula the oral caloric intake was not significantly different from the mean daily deficit in calories given by fistula.

Hill and Dansky ('50, '54) and Dansky and Hill ('51), who based their findings on calculated productive energy values, and Peterson et al. ('54), whose calculations involved estimated metabolizable energy values, have reported a close association between the feed intake of chicks and the available energy content of the ration. Fisher and Weiss ('56) noted that on some rations chicks ate to satisfy their energy requirements, but also reported that fibre *per se* stimulated a greater feed intake.

A close association between the variance in the food intake of weanling rats and the A.D.E.[2] content of the ration was reported by Sibbald et al. ('56, '57a) while Finlayson and Baumann ('56) noted that the addition of as much as 30% of cellulose to the rations of growing rats did not appreciably

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[2] Throughout this report the following abbreviations are employed: A.D.E. apparent digestible energy and A.D.N. apparent digestible nitrogen.

influence caloric intake. When Sibbald et al. ('57b) fed protein-free rations, containing a mixture of amino acids and diammonium citrate, to weanling rats it was reported that 93% of the variance in the volume of food consumed was associated with the reciprocal of the A.D.E. per 100 ml of food, and that density *per se* did not influence food intake.

Since Hoppe (1856) reported that carbohydrate ingestion lowered the nitrogen excretion of dogs many workers have demonstrated the protein-sparing action of energy (Bosshardt and Barnes, '46; Leverton et al., '51; Rosenthal and Allison, '51; Swanson, '51; and Calloway and Spector, '55). Meyer ('56) noted that as the indigestible portion of the food increased the total fecal nitrogen excretion of weanling rats also increased. Sibbald et al. ('56) observed that 69% of the variation in nitrogen retention was associated with A.D.E. consumption, while Sibbald et al. ('57a) reported that the relationship between the percentage of A.D.E. retained and the A.D.E. per gram of A.D.N. was curvilinear in nature and found an association of 87% between the variables. In a report of a later experiment, in which a mixture of amino acids and diammonium citrate was the nitrogenous source, Sibbald et al. ('57b) noted a similar relationship, the association between the variables being 65%.

The experiments reported herein were designed to study the influence of the A.D.E. content of the ration on both the weight and volume of food consumed. The range of ration A.D.E. levels was greater than that used in previous experiments in order to attempt to determine the physiological limits of food intake. The influence of the nature of the nitrogen source on A.D.E. consumption was studied as was the relationship between the A.D.E. : A.D.N. ratio of the food and the percentage of A.D.N. retained for the different nitrogen sources.

METHODS

Two male and two female weanling rats were allotted to each of the 37 experimental rations. The composition of these

rations is presented in tables 1 and 2. In the formulation of the rations 6 different nitrogen sources were used; these were: nitrogen source "A" (a casein: lactalbumin mixture supplemented with amino acids Sibbald et al., '56, '57a), fish meal, meat scrap, solvent extracted soybean oil meal, casein and corn gluten. Variation in the levels of fibre [3] or the nitrogen sources or both were made at the expense of the sucrose portion of the rations. All rations contained vitamin and mineral supplements.

The management of the experimental animals and the collection and analysis of samples followed the methods outlined by Sibbald et al. ('57a). The experimental period of 7 days followed a 7-day ration acclimatization period. Statistical analyses were conducted using data for individual animals, the use of group means being limited to tables. Since many of the variables studied were influenced by body weight it was necessary to remove variation associated with this factor prior to determining the magnitude of the various relationships under consideration. This was accomplished by expressing the variables on a 100 gm body weight basis. The body weight, referred to, was the mean of the initial and final body weights of individual rats during the experimental period.

RESULTS

Food consumption. The principal mean values relative to food consumption, obtained in these experiments, are recorded in tables 1 and 2. It will be noted that an increase in the Alphacel content of a ration was associated with a decrease in both the density and the A.D.E. content of the food. Within any group of rations, based on a single nitrogen source, the A.D.E. intake per 100 gm body weight was remarkably uniform; an exception to this was noted for rats consuming rations containing meat scrap. It would appear, therefore, that a decrease in the A.D.E. content of a ration was compensated for by an increase in food consumption.

[3] Alphacel, "non-nutritive cellulose." Nutritional Biochemicals Corporation, Cleveland, Ohio.

TABLE 1

Composition of rations containing nitrogen source "A" with additional data relating to their consumption by weanling rats

RATION	RATION COMPOSITION [1]			FOOD ANALYSIS			A.D.E./100 GM FOOD	A.D.E./100 ML FOOD	FOOD CONSUMPTION /100 GM BODY WT.		A.D.E./ 100 GM BODY WT.	BODY WT. OF RATS
	Fibre [2]	Nitrogen source "A" [3]	Sucrose	Density	Gross nitrogen	Gross energy						
	%	%	%	*gm/ml*	*mg/ 100 gm*	*Cal./ 100 gm*	*Cal.*	*Cal.*	*gm*	*ml*	*Cal.*	*gm*
1	30	20.3	39.7	0.357	2708	423	298	106	98	274	292	85
2	40	20.3	29.7	0.307	2791	424	260	80	111	364	290	82
3	45	20.3	24.7	0.280	2732	427	245	69	122	434	298	84
4	50	20.3	19.7	0.273	2760	426	227	62	132	485	301	82
5	55	20.3	14.7	0.257	2719	432	204	52	155	602	315	68
6	60	20.3	9.7	0.243	2680	430	186	45	161	662	299	65
7	65	20.3	4.7	0.232	2612	426	167	39	179	768	299	55
8	0	10.2	79.8	0.662	1304	413	401	265	86	130	345	54
9	10	10.2	69.8	0.562	1336	410	361	202	84	150	303	68
10	20	10.2	59.8	0.420	1362	414	330	138	94	224	310	73
11	30	10.2	49.8	0.356	1328	412	292	104	102	286	298	79
12	10	11.4	68.6	0.529	1488	416	365	193	87	165	318	71
13	20	11.4	58.6	0.429	1546	416	332	142	88	205	292	78
14	30	11.4	48.6	0.361	1512	416	297	107	102	282	303	85
15	20	12.7	57.3	0.410	1650	422	340	140	92	224	313	81
16	30	12.7	47.3	0.345	1702	419	300	103	99	287	297	90
17	20	14.0	56.0	0.422	1798	419	334	140	88	210	294	77
18	30	14.0	46.0	0.341	1835	424	306	104	102	300	312	74
19	10	16.5	63.5	0.524	2156	421	374	194	80	154	299	85
20	20	16.5	53.5	0.417	2232	422	340	141	90	217	306	84
21	10	19.0	61.0	0.513	2401	427	380	194	82	161	312	78
22	30	19.0	41.0	0.344	2544	428	301	103	99	289	298	88

[1] All rations contained 4% salts (Jelinek et al., '52), 5% Mazola oil and 1% vitamin mix (Sibbald et al., '56).

[2] Alphacel "non-nutritive cellulose." Nutritional Biochemical Corporation, Cleveland, Ohio.

[3] Nitrogen source "A" was a casein:lactalbumin mixture supplemented with amino acids (Sibbald et al., '56, '57a).

TABLE 2

Composition of rations containing nitrogen sources other than "A" with additional data relating to their consumption by weanling rats

RATION [2]	RATION COMPOSITION [1]			FOOD ANALYSIS			A.D.E./100 GM FOOD	A.D.E./100 ML FOOD	FOOD CONSUMPTION /100 GM BODY WT.		A.D.E./ 100 GM BODY WT.	BODY WT. OF RATS
	Fibre	Nitrogen source	Sucrose	Density	Gross nitrogen	Gross energy						
	%	%	%	*gm/ml*	*mg/ 100 gm*	*Cal./ 100 gm*	*Cal.*	*Cal.*	*gm*	*ml*	*Cal.*	*gm*
F1	10	10.0	75.0	0.701	1272	386	336	235	77	110	259	52
F2	23	10.0	62.0	0.588	1243	384	290	169	91	156	264	56
F3	37	10.0	48.0	0.470	1232	383	234	110	117	249	274	64
M1	12	15.0	68.0	0.687	1015	371	306	210	63	92	193	36
M2	25	15.0	55.0	0.562	1254	374	274	154	90	160	247	41
M3	38	15.0	42.0	0.454	1262	371	210	95	83	183	174	44
S1	13	15.0	67.0	0.687	1176	382	321	221	86	125	276	54
S2	26	15.0	54.0	0.576	1201	384	273	156	98	172	268	58
S3	39	15.0	41.0	0.440	1241	384	226	100	119	270	269	62
C1	22	7.5	60.5	0.420	1144	415	327	138	80	190	262	52
C2	36	7.5	46.5	0.340	1183	415	278	94	92	271	256	62
C3	48	7.5	34.5	0.292	1456	414	225	66	130	443	292	75
G1	23	8.0	59.0	0.407	1156	413	315	129	75	183	236	44
G2	36	8.0	46.0	0.313	1210	417	276	86	86	274	237	49
G3	49	8.0	33.0	0.275	1171	414	217	60	106	386	230	47

[1] All rations contained 4% salts (Jelinek et al., '52) and 1% vitamin mix (Sibbald et al., '56). Rations prefixed C or G also contained 5% Mazola oil.

[2] Prefixes refer to nature of nitrogen source: F = white fishmeal, M = meat scrap, S = solvent extracted soybean oil meal, C = casein and G = corn gluten.

The mean A.D.E. consumption per 100 gm body weight for each of the 6 nitrogen sources was as follows: nitrogen source "A" 304 Cal., fish meal 266 Cal., soybean oil meal 271 Cal., casein 270 Cal., corn gluten 234 Cal. and meat scrap 205 Cal. Analysis of variance indicated a highly significant ($P < 0.01$) difference in the A.D.E. intake of rats between nitrogen sources with an L.S.D. of 29 Cal. at the 1% level of significance. In the aforementioned analysis of variance and in the calculation of the L.S.D., data from only three of the 22 rations containing nitrogen source "A" were employed; this allowed for a balanced design with equal numbers from each of the 6 nitrogen sources. The rations represented were numbers 16, 18 and 19 (table 1) which were drawn at random.

A summary of the correlations between food consumption and the reciprocal of the A.D.E. content of the food is presented in table 3. It will be noted that correlations between the volume of food consumed and the reciprocal of the A.D.E. per 100 ml of food yielded higher r values than those obtained when the weight of food consumed was correlated with the reciprocal of the A.D.E. per 100 gm of food. This apparently was not the direct result of a change in food density for when rations containing nitrogen source "A" were grouped according to density no difference in the A.D.E. consumption per 100 gm body weight was detected by analysis of variance.

The correlation between the volume of food consumed per 100 gm body weight and the reciprocal of the A.D.E. per 100 ml of food, for rats receiving rations containing nitrogen source "A", yielded an r value of 0.992 at 86 D.F. This indicates an almost perfect straight line relationship between the two variables. Therefore, it may be assumed that even on rations containing as much as 65% of Alphacel, with as few as 39 Cal. of A.D.E. per 100 ml of food, rats were able to eat to satisfy their energy requirements.

Nitrogen retention. Mean data relative to nitrogen retention are presented in tables 4 and 5. The data of table 4

TABLE 3

A summary of correlations between food intake and the A.D.E. content of the food

	D.F.	r_{yx} [1]	SIG.	r_{y1x1} [2]	SIG.
Nitrogen source "A"	86	0.987	0.01	0.992	0.01
Fish meal	10	0.967	0.01	0.985	0.01
Meat scrap	10	0.416	N.S.	0.830	0.01
Soybean oil meal	10	0.832	0.01	0.971	0.01
Casein	10	0.905	0.01	0.964	0.01
Corn gluten	10	0.882	0.01	0.962	0.01

[1] r_{yx} = the correlation between the weight of food consumed per 100 gm body weight and the reciprocal of the A.D.E. per 100 gm of food.

[2] r_{y1x1} = the correlation between the volume of food consumed per 100 gm body weight and the reciprocal of the A.D.E. per 100 ml of food.

TABLE 4

Mean data relating to the nitrogen retention of rats receiving rations containing nitrogen source "A" [1]

RATION	NITROGEN CONSUMED /100 GM BODY WT.	NITROGEN DIGESTED	A.D.N. RETAINED	A.D.E./GM A.D.N. IN FOOD	FOOD DIGESTIBILITY
	mg	%	%	*Cal.*	%
1	2691	89	57	124	68
2	3271	87	53	107	58
3	3518	86	49	104	53
4	3655	86	48	96	49
5	4264	84	40	90	44
6	4482	82	35	84	37
7	5482	78	31	82	32
8	1125	97	78	318	98
9	1124	91	85	296	88
10	1282	88	90	276	79
11	1354	88	86	251	70
12	1302	92	88	268	88
13	1360	91	88	236	79
14	1536	88	85	222	70
15	1513	90	83	228	79
16	1669	89	79	198	70
17	1590	90	79	206	79
18	1887	88	72	188	70
19	1736	93	78	187	88
20	2007	91	73	168	79
21	1976	92	70	172	88
22	2534	88	62	134	68

[1] The composition of these rations is presented in table 1.

indicate that as the indigestible portion of the ration increased the apparent digestibility of the food nitrogen decreased. A similar trend was exhibited by the data relative to rations containing fish meal and soybean oil meal listed in table 5.

A summary of the correlations between the percentage of A.D.N. retained and the A.D.E. per gram of A.D.N. for the 6 nitrogen sources is presented in table 6. The association between the percentage of A.D.N. retained and the A.D.E. per gram of A.D.N. in the food, for rats fed rations containing nitrogen source "A", yielded a highly significant r value of 0.894 at 86 D.F. A test for curvilinearity of regression demonstrated a significantly ($P < 0.01$) improved relationship; a highly significant R value of 0.955 at 85 D.F. indicates an association of 91% between the variables. The quadratic regression equation for the curvilinear relationship is:

$$Y = -18.9 + 0.7946X - 0.001500\,X^2$$

where Y is the percentage of A.D.N. retained and X the A.D.E. per gram of A.D.N. in the food. This equation yielded a maximum value for Y of 86.3% when X equalled 265 Cal. of A.D.E. per gram of A.D.N. The original standard deviation for the data on percentage of A.D.N. retained was 18.5% and the standard error of estimate for deviations from curvilinear regression ($s_{y.x}$), which indicates the variation remaining after removing that associated with the A.D.E. : A.D.N. ratio of the food, was 5.5%.

The limited number of rations employed prevented the derivation of similar quadratic equations for the other 5 nitrogen sources. The data of table 5 show very little variation in the percentage of A.D.N. retained relative to the changes in the levels of A.D.E. per gram of A.D.N. in the food. The low r values resulting from correlations between the A.D.E. per gram of A.D.N. and the percentage of A.D.N. retained for the different nitrogen sources together with the almost horizontal regression lines of figure 1 indicate that the levels of A.D.E. per gram of A.D.N. employed resulted in percentages of A.D.N. retained which were close

to the maxima, that is within the bounds of the plateaux of the curves. An exception to this was meat scrap, but this may be explained by the extremely high level of A.D.E. per gram of A.D.N. (482 Cal.) which occurred in ration M1. This high

TABLE 5

Mean data relating to the nitrogen retention of rats receiving rations containing nitrogen sources other than "A"[1]

RATION	NITROGEN CONSUMED /100 GM BODY WT.	NITROGEN DIGESTED	A.D.N. RETAINED	A.D.E./GM A.D.N. IN FOOD	FOOD DIGESTIBILITY
	mg	%	%	*Cal.*	%
F1	988	80	65	334	86
F2	1142	78	74	300	76
F3	1435	75	73	252	62
M1	638		−32	482	80
M2	1120	[illegible]	27	323	70
M3	1050	60	0	277	54
S1	1015	78	62	347	84
S2	1170	76	62	300	71
S3	1476	71	66	256	60
C1	906	85	66	337	77
C2	1086	79	70	298	65
C3	1882	86	76	180	52
G1	866	78	25	348	75
G2	1041	80	25	286	63
G3	1225	74	26	251	49

[1] The composition of these rations is presented in table 2.

TABLE 6

A summary of correlations between the percentage A.D.N. retained and the A.D.E. per gram of A.D.N.

NITROGEN SOURCE	D.F.	CORRELATION	SIG.
"A"	86	0.894	0.01
"A"	85	0.955[1]	0.01
Fish meal	10	−0.336	N.S.
Meat scrap	10	−0.929	0.01
Soybean oil meal	10	−0.300	N.S.
Casein	10	−0.583	0.05
Corn gluten	10	0.142	N.S.

[1] This is the multiple correlation value and takes account of the curvilinear association between the variables.

level of A.D.E. resulted in a percentage of A.D.N. retained which was considerably displaced to the right of the maximum of the curve and allowed a significant negative correlation ($r = -0.929$ at 10 D.F.) to occur. As the data relating to meat scrap were atypical, the regression line relative to this

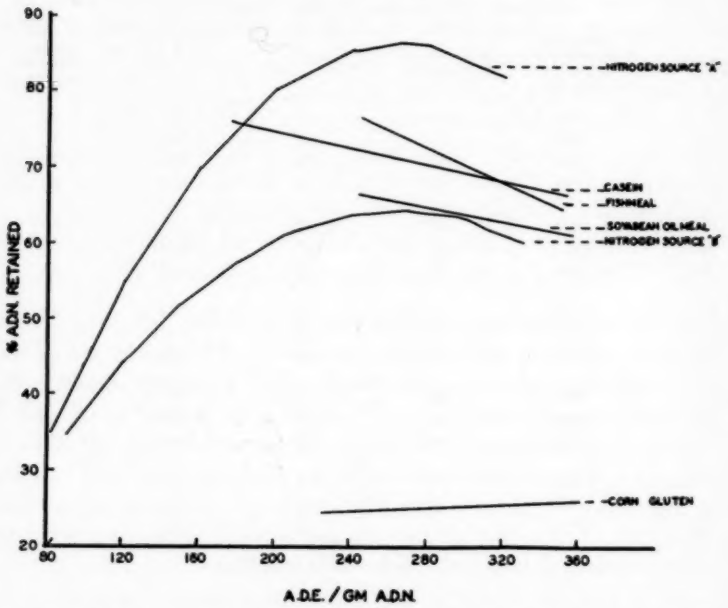

Fig. 1 The influence of A.D.E. on A.D.N. retention (curve for nitrogen source "B" from Sibbald et al., '57b).

nitrogen source has been omitted from figure 1. Although the data are not conclusive it would appear that the maximum percentage of A.D.N. retained probably occurred when the A.D.E. per gram of A.D.N. fell between 250 and 300 Cal. This is supported by the positions of the maxima of the two curvilinear regression lines included in figure 1 where more data were available.

An interesting feature of figure 1 is the relative values of the approximate maximum percentages of A.D.N. retained. If these maxima indicate the quality of the nitrogen sources then it will be noted that nitrogen source "A" was superior to fish meal, casein, soybean oil meal and nitrogen source "B" which in turn were superior to corn gluten. The data relative to nitrogen source "B" are derived from an earlier report (Sibbald et al., '57b).

DISCUSSION

Food consumption. The A.D.E. intake of the weanling rats used in these experiments appeared to be influenced by the nature of the nitrogen source. Below, the nitrogen sources are listed in the approximate order of quality and compared with the average A.D.E. intake per 100 gm body weight of the rats.

Nitrogen source "A"	304 Cal.
Fishmeal	266 Cal.[4]
Soybean oil meal	271 Cal.[4]
Casein	270 Cal.[4]
Nitrogen source "B"[5]	270 Cal.[4]
Corn gluten	234 Cal.

It would appear that as the quality of the nitrogen source increases a corresponding increase occurs in the A.D.E. intake per 100 gm body weight.

It will be remembered that in the analysis of variance between the A.D.E. intake per 100 gm body weight of the rats only rations 16, 18 and 19 of the 22 rations containing nitrogen source "A" were involved in the calculations. The average gross nitrogen content of these rations was 1,897 mg per 100 gm which is considerably higher than the nitrogen content of the rations based on the other nitrogen sources. However, examination of the data of table 1 indicates the improbability that the level of nitrogen in the rations affected A.D.E. intake.

[4] Nitrogen sources of approximately the same quality.
[5] Data from Sibbald et al. ('57b).

Further evidence that nitrogen level of the ration does not directly influence food, and therefore A.D.E., consumption has previously been presented by Sibbald et al. ('57a, b).

Fisher and Weiss ('56) reported that fibre *per se* is an important factor, stimulating the feed intake of chicks independently of the energy level of the ration. This may be interpreted to suggest that fibre *per se* increases the intake of available energy. However, rats on ration 8 of this experiment, the only ration containing no Alphacel, consumed an average of 345 Cal. of A.D.E. per 100 gm body weight compared to a mean of 302 Cal. for rats receiving rations containing Alphacel and based on the same nitrogen source. These results, though not conclusive, would indicate that fibre tends to depress the energy intake of weanling rats. Fisher and Weiss ('56) derived their energy values from computations involving the Atwater factors for metabolizable energy. In a footnote it was pointed out that Anderson and Hill ('55) have derived a factor for tallow, in chick nutrition, which is considerably lower than that presented by Atwater. If the factor of Anderson and Hill ('55) had been used in the calculations it is doubtful whether fibre as such would have been found to exert a very significant influence on either feed or energy intake. If the factor is more correct than that of Atwater then the variation between the approximately isocaloric rations of Fisher and Weiss ('56) might be of sufficient magnitude to explain part of the stimulation of feed and energy intake, attributed to fibre, on the basis of a variation in growth rate resulting from differences in the protein: energy ratio. Peterson et al. ('54) reported a stimulation in the feed consumption of chicks when fibre replaced some of the glucose in a ration, but explained this stimulation as being a function of the greater growth-promoting effect of the fibre-containing rations resulting from the increased ratio of protein to other digestible nutrients. It would appear that to obtain satisfactory information regarding the influence of available energy and fibre on food consumption, calculations should be based on the results of actual energy determinations and not on

calculated figures. The latter are subject to several sources of error such as those involved in mixing the rations and the variation in digestibility between individual animals.

Changes in density were not found to influence the A.D.E consumption of weanling rats. However, changes in density were closely associated with changes in the A.D.E. content of the food. For this reason a measure of the correlation between the volume of food consumed per 100 gm body weight and the reciprocal of the A.D.E. per 100 ml of food yields a higher r value than that between the weight of food consumed per 100 gm body weight and the reciprocal of the A.D.E. per 100 gm of food. This supports the findings of Sibbald et al. ('57b).

It has been pointed out that the A.D.E. consumption, per 100 gm body weight, of weanling rats did not show any significant variation when the A.D.E. per 100 ml of food varied from 39 to 265 Cal. This indicates that the maximum possible food consumption was not reached even when a ration contained 65% of Alphacel.

Nitrogen retention. The results, relative to the nitrogen retention of rats receiving nitrogen source "A", have demonstrated the curvilinear relationship between the percentage of A.D.N. retained and the A.D.E. per gram of A.D.N. The association between the variables was 91% and a maximum of 86.3% of the A.D.N. was retained when the food contained 265 Cal per gram of A.D.N. In an earlier experiment (Sibbald et al., '57a) in which fewer rations were involved but in which the same nitrogen source was employed the corresponding figures were 87%, 89.3% and 277 Cal. of A.D.E. per gram of A.D.N. The variation between the results of the two experiments may be explained by the considerably greater range of the values of A.D.E. per gram of A.D.N. employed in this experiment, which allowed for the formulation of a more reliable curve.

The nitrogen retention data relative to rats receiving rations containing nitrogen sources other than "A" do not allow any definite conclusions to be drawn, because of the small number

of rations involved. However, there is an indication that the maximum percentage of A.D.N. retained for each nitrogen source occurred when the A.D.E. per gram of A.D.N. of the food fell within the range of 250 to 300 Cal. Previous experiments have shown maxima to occur in this range (Sibbald et al., '57a, b). It seems worthwhile to continue studies into the relationship between the A.D.E. per gram of A.D.N. of the food and the A.D.N. retention for, if the maxima always occur in a narrow range for each physiological process, e.g. growth, gestation, lactation etc., the formulation of satisfactory rations would be simpler and would avoid the excessive and uneconomical use of either protein or energy, the two most expensive components of any practical ration.

SUMMARY

Variations in the food consumption of weanling rats, fed rations containing varying nitrogen sources, were largely associated with the apparent digestible energy (A.D.E.) content of the rations. A significant difference in the A.D.E. consumption of rats between nitrogen sources was attributed to the quality of the nitrogen sources. The higher the quality of the nitrogen source the greater the A.D.E. consumption. Evidence was presented to suggest that fibre in the diet tends to depress A.D.E. consumption. The higher physiological limit of food consumption was not attained even when the ration contained 65% of Alphacel.

The nitrogen retention data stressed the importance of the ratio of apparent digestible energy to apparent digestible nitrogen (A.D.N.) of the food in controlling the percentage of A.D.N retained. It is possible that the optimum level of A.D.E. per gram of A.D.N. for all nitrogen sources studied (in respect to the growth of weanling rats) was within the range of 250 to 300 Cal.

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THE VANDERBILT COOPERATIVE STUDY OF MATERNAL AND INFANT NUTRITION [1]

X. ASCORBIC ACID

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INTRODUCTION

The basic findings of the Vanderbilt cooperative study of maternal and infant nutrition have been reported (Darby et al., '53a, b; McGanity et al., '54), but little of the detail of the interrelationships of various factors was included. Analyses of the interrelationships of biochemical and dietary data in large groups of persons are rare. Such analyses are necessary, however, to the soundest interpretation of nutritional data. Accordingly, we have studied each of several nutrient groups separately and explored numerous possible relations. This paper deals with the dietary intake and the serum levels of ascorbic acid, the variation in each of these with time of gestation, season of the year, and with age, parity, height, weight, and physical findings of the mother. The variation in serum vitamin C with intake levels and the

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relation of both intake and serum levels to the course and outcome of pregnancy and to lactation are also explored. The data are for 2,129 consecutively encountered pregnant women, the outcome of the pregnancy being observed during the course of the investigation. The methods employed have been described in the earlier reports.

OBSERVATIONS AND DISCUSSION

Intake levels. The distribution of recorded intakes of vitamin C is given in table 1. This is based on a one-week diet record obtained from each patient once during each trimester of pregnancy. A wide range in reported intake is apparent, and there occurs a slight overall decrease in intake level from the first to the third trimester.

Median consumption in the study group was lower than the allowances recommended by the Food and Nutrition Board ('53) of 70 mg per day for adult women and 100 mg per day during the third trimester of pregnancy. Of those who had diet records for both the second and third trimesters, 14% reported daily intakes of less than 40 mg of vitamin C on both occasions, and 2% reported intakes of less than 20 mg on both records. Only 15% had intakes of 80 mg or more on both records.

Serum levels. As previously reported (Darby et al., '53b), serum vitamin C levels declined during pregnancy and were lowest at the time of the postpartum examination. Distributions are shown in table 1. Postpartum values were clearly lower for lactating than for non-lactating women, but a corresponding difference between these groups was not observed during pregnancy. In other words, the low values of serum ascorbic acid which are associated with lactation appear to be a reflection of lactation rather than the result of a pre-existing difference between those who lactated and those who did not.

Serum vitamin C levels during pregnancy and lactation have been studied by many investigators (Ingalls et al., '38;

TABLE 1

Distribution of dietary intakes and serum levels of vitamin C by period of the reproductive cycle

INTAKE				SERUM LEVELS					
Intake of vitamin C	First trimester	Second trimester	Third trimester	Serum vitamin C	First trimester	Second trimester	Third trimester	Postpartum [1]	
								Lactating	Non-lactating
mg/day	*Number of women*			*mg/100 ml*	*Number of women*				
0– 19	12	76	174	Under 0.20	72	315	638	510	235
20– 39	49	205	374	0.20–0.39	80	289	458	158	179
40– 59	59	308	392	0.40–0.59	53	236	316	67	84
60– 79	61	244	308	0.60–0.79	56	217	235	51	58
80– 99	34	174	180	0.80–0.99	32	146	163	24	49
100–119	33	91	104	1.00–1.19	23	95	89	13	22
120–139	13	69	68	1.20–1.39	17	48	41	2	7
140–159	9	27	24	1.40–1.59	1	17	12	1	2
160–179	5	14	15	1.60–1.79	3	3	5	1	1
180–199	2	4	10	1.80–1.99	1	1	2		
200 and over	1	9	16	2.00 and over		2	1		
Total subjects	278	1221	1665	Total subjects	338	1369	1960	827	637
Median intake (mg/day)	66	62	54	Median (mg/100 ml)	0.46	0.47	0.35	0.16	0.29

[1] Postpartum data do not include women who had abortions or twins.

Teel et al., '38; Snelling and Jackson, '39; Lund and Kimble, '43; Anderson et al., '46; Young et al., '46; Munks et al., '47; Hoch and Marrack, '48; and Moyer et al., '54). The present analysis considers the influence of both intake levels and period of gestation on serum levels and hence offers an opportunity to extend our interpretation of such data.

Seasonal pattern. Seasonal variation occurred in both the intake and the serum vitamin C content (figs. 1, 2). The months of lowest recorded intake during the second trimester were January, February, June, July and September, and in the third trimester June, July, August, September and October. Serum vitamin C levels were lowest in the period of February through June. In other words, the season (July-September) of highest blood levels was a period when recorded intakes were relatively low. This inconsistency will be considered again in a later section. We have no explanation as to why the variation in intake with season was not identical for the second and third trimesters.

Correlation of values obtained for the same patient in different trimesters. In nutrition surveys or in the clinical assessment of nutriture the use of a one-week diet record or of a single laboratory determination is based on the assumption that each of these gives a reasonably reliable measure of the intake or serum level for the period in question. If the values vary greatly from one time to another, then a single record is obviously inadequate. It is of interest, therefore, to compare the records of the same patient in different trimesters in order to observe the constancy of findings over a time interval.

There is a moderate degree of correlation between the recorded vitamin C intake of the same individuals in different trimesters, as well as between serum vitamin C levels taken at different times. Tables 2 and 3 typify the degree of these correlations. Although there were some individuals having high values at one time and low values at another, the majority of cases showed reasonably good agreement. However, because of the large differences observed in some cases, it seemed

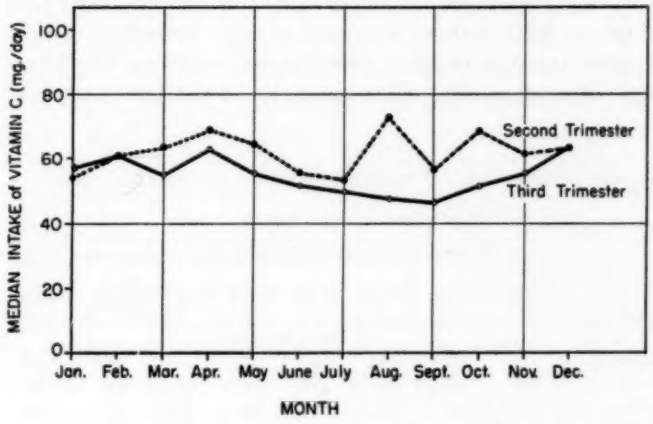

Fig. 1 Median dietary intake of vitamin C by month.

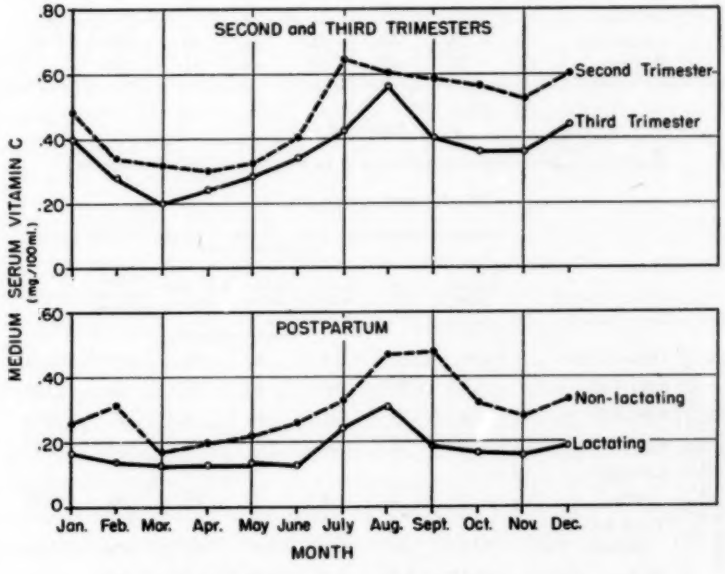

Fig. 2 Median serum levels of vitamin C by month.

likely that a comparison between those individuals who had either low or high values on each of two occasions might yield sharper contrasts than comparison made on the basis of a single determination. Consequently, in the latter part of

TABLE 2

Relation of dietary intakes of vitamin C in second and third trimesters

(Winter and spring seasons)

THIRD TRIMESTER INTAKE (MG/DAY) (SPRING SEASON)	SECOND TRIMESTER INTAKE (MG/DAY) (WINTER SEASON)							
	Under 20	20–39	40–59	60–79	80–99	100 and over	Total number patients	Median
	Number of cases							
Under 20	3	6	8	3	2		22	45
20–39	6	16	16	11	4	3	56	48
40–59	2	8	12	11	12	6	51	66
60–79	2	3	10	6	10	9	40	77
80–99	2	2	6	3	6	6	25	77
100 and over		1	6	7	6	16	36	93
Total no. patients	15	36	58	41	40	40	230	63
Median	35	35	48	52	64	87	55	

TABLE 3

Relation of serum levels of vitamin C in second and third trimesters

(Winter and spring seasons)

THIRD TRIMESTER LEVELS OF SERUM VITAMIN C (MG/100 ML) (SPRING SEASON)	SECOND TRIMESTER LEVELS OF SERUM VITAMIN C (MG/100 ML) (WINTER SEASON)						
	Under 0.20	0.20–0.39	0.40–0.59	0.60–0.79	0.80 and over	Total number patients	Median
	Number of cases						
Under 0.20	51	27	12	8	8	106	0.21
0.20–0.39	10	22	17	6	9	64	0.40
0.40–0.59	10	9	12	8	8	47	0.48
0.60–0.79	3	3	4	7	4	21	0.61
0.80 and over	3	3	4	5	26	41	0.96
Total no. patients	77	64	49	34	55	279	0.40
Median	0.15	0.25	0.35	0.48	0.72	0.30	

this paper groups having "consistenly" low, medium or high intakes or serum levels of vitamin C are reported.

Correlation of serum vitamin C with vitamin C intake. Serum levels of vitamin C vary with recorded intake, but the degree of correlation is not high (table 4). However, there were distinct differences in average values of serum vitamin C for groups at different intake levels. In each trimester daily intakes were grouped as follows: less than 40 mg, 40 to 79 mg, 80 to 119 mg, and 120 mg and over. Median serum vitamin C values were determined for each intake group, postpartum values being studied in relation to third trimester intakes. Cases were limited to those for whom the interval between the diet record and the serum determination was not more than three weeks (except for the postpartum group), and subdivision of the data by season (on the basis of laboratory values) was made as follows: winter (January through March), spring (April through June), summer (July through September), and fall (October through December). Table 5 gives median serum vitamin C values for the third trimester. Data for second trimester and postpartum show similar differences. Median serum levels increased with increasing intake. In addition, clear seasonal differences in the levels in the blood for similar recorded intakes were present, except possibly in the first trimester. For a given nutrient level, serum vitamin C values were highest in summer followed by fall, winter and spring, in that order. These seasonal differences were statistically significant except those between winter and spring. This same phenomenon was reflected in the difference in seasonal patterns between nutrient and serum vitamin C levels noted above. Among the influences which may contribute to these seasonal inconsistencies are seasonal variation in the vitamin C content of some foods (for which no allowance was attempted in calculations), and seasonal shifts in the composition of the intake groups. It is also possible that there is seasonal variation in vitamin C requirements, or in certain metabolic processes which affect serum levels of vitamin C.

Correlation of serum vitamin C with other laboratory determinations. Serum vitamin C levels were positively correlated with serum carotene levels and to a slight extent with serum vitamin A levels.[2] Intakes of vitamin C and carotene were also correlated. This is not unexpected, inasmuch as dietary

TABLE 4

Relation between nutrient intake and serum levels of vitamin C, winter season, third trimester

SERUM VITAMIN C	INTAKE OF VITAMIN C (MG/DAY)					
	Under 40	40–79	80–119	120 and over	Total	Median intake
mg/100 ml	*Number of cases*					
Under 0.39	107	91	45	14	257	47
0.40–0.79	26	51	22	14	113	66
0.80–1.19	8	19	18	10	55	81
1.20 and over	4	5	5	7	21	95
Total patients	145	166	90	45	446	57
Median serum level	0.21	0.36	0.40	0.62	0.33	

TABLE 5

Median levels of serum vitamin C in relation to nutrient intake, by season, third trimester

INTAKE	MEDIAN SERUM VITAMIN C				NUMBER OF CASES			
	Winter	Spring	Summer	Fall	Winter	Spring	Summer	Fall
mg/day	*mg/100 ml*							
Under 40	0.21	0.20	0.36	0.30	145	133	125	109
40–79	0.36	0.34	0.52	0.44	166	191	141	145
80–119	0.40	0.31	0.61	0.54	90	78	41	58
120 and over	0.62	0.57	0.80	0.52	45	31	14	35
All intake levels	0.33	0.29	0.46	0.40	446	433	321	347

sources of carotene and of ascorbic acid are often the same — especially greens of various sorts.

Serum vitamin C levels were also positively correlated with urinary excretion values, after a test dose, of niacin and, to perhaps a slight degree, of riboflavin and thiamine.

[2] Martin, Bridgforth, McGanity and Darby, unpublished data.

These correlations seem to reflect a corresponding correlation in intakes.

There is no apparent relationship between ascorbic acid level and serum proteins.

A positive relationship between certain hematological values and the intakes and serum levels of vitamin C will be discussed in the sections on "consistent" groups.

Parity and other factors. With increasing parity there occurred a decrease in both vitamin C intakes and serum levels. Data for the third trimester are shown in table 6. The trend in vitamin C level in the serum associated with

TABLE 6

Median intakes and serum vitamin C levels by parity, third trimester

PARITY	DAILY INTAKE	NUMBER OF CASES	MEDIAN SERUM VITAMIN C	NUMBER OF CASES
	mg		*mg/100 ml*	
0	56	546	0.42	621
1–2	56	645	0.36	752
3–4	52	230	0.28	274
5 and over	48	213	0.24	272

parity seems to be over and above that associated with differences in recorded intakes. It may reflect the depleting effect of successive pregnancies (and lactation). The present data, however, do not permit a decisive analysis of this possibility.

No association was found between serum ascorbic acid content and age, height, or weight of mother.

Vitamin C in relation to physical findings and disease conditions in the mother. The study and interpretation of data on physical findings were complicated by the differences among examiners in the frequency with which a given condition was diagnosed (McGanity et al., '54). Nevertheless, two physical findings, gingival changes and edema, seemed possibly to exhibit some relationship to vitamin C nutriture.

The percentage of cases with gingival lesions (red or swollen gums or pyorrhea) was 23, 24, 28, and 27 for successive periods from the first trimester through the postpartum. The incidence of gingival changes was higher among women with low concentrations of vitamin C in their serum but the differences were not large. Gum findings ran high in the women of higher parities, averaging about 40% for those of parity three and over, and there was little relation to dietary intakes or serum levels of vitamin C in this group. At the lower parities there was some evidence of a relation. For example, when groups with "consistent" levels of serum vitamin C (see below) were compared, the percentages with gingival findings in the second trimester were found to be 21, 17 and 8 for low, medium and high concentrations in the serum, respectively, for parity two and under. These differences are significant ($P = 0.002$). There were not significant differences in the other trimesters or postpartum.

A significant relationship between gingival findings and intake of vitamin C appeared only when groups with "consistently" low, medium, and high vitamin C intakes (see below) were compared on the basis of the number having gum findings on two or more physical examinations. Among women of parity two or less with "consistently" low vitamin C intakes, 19% had gum findings on at least two examinations. Among the "consistently" medium and high intake groups the corresponding percentages were 14 and 9, respectively. Thus it seems likely that vitamin C may play a role in gingival lesions, but that it is not of major importance.

Linghorne et al. ('46) in an experiment in which dietary intake was controlled, showed a relationship between the occurrence of gingivitis and the intake of vitamin C, but they found no effect of the administration of vitamin C on pre-existing gingivitis. Our data are in agreement with these findings. While ascorbic acid nutriture may be a factor in the development of gingivitis in young mothers of low parity, other factors are primarily responsible for the continuation of gingivitis observed among the older multiparae.

The number of women having edema increased from 2.4% in the first trimester to 10.8% in the third and returned postpartum to 2.1%. Only in the second trimester was there a significant relationship between serum level of ascorbic acid and edema. We were unable to detect a relationship between intake of vitamin C and the occurrence of edema in any trimester, even among the "consistent" groups. We, therefore, conclude that vitamin C is not an important factor in the occurrence of edema in our study group.

It was found (McGanity et al., '54) that women with anemia during pregnancy had lower intakes and serum levels

TABLE 7

Frequency of occurrence of single, live-born premature infants according to intake and serum levels of vitamin C in the third trimester of pregnancy

VITAMIN C INTAKE	TOTAL CASES	PREMATURES		SERUM VITAMIN C	TOTAL CASES	PREMATURES	
mg/day		*No.*	*%*	*mg/100 ml*		*No.*	*%*
Under 20	170	12	7.1	Under 0.20	622	34	5.5
20–39	371	10	2.7	0.20–0.39	449	19	4.2
40–59	385	8	2.1	0.40–0.79	537	17	3.2
60–99	475	14	2.9	0.80 and over	311	11	3.5
100 and over	233	6	2.6				
Total	1634	50	3.1	Total	1919	81	4.2

of vitamin C than the rest of the study group. Also, women with disease conditions complicating pregnancy [3] had somewhat lower intakes and serum levels during pregnancy. Relation of vitamin C to hematologic values will be considered again later in the present paper.

Vitamin C in relation to the course and outcome of pregnancy. As reported in a previous paper (McGanity et al., '54), mothers of premature [4] infants had lower serum vitamin C levels and lower intakes of vitamin C during pregnancy,

[3] Tuberculosis, venereal disease, endocrine disorders, heart disease, genitourinary diseases, and miscellaneous other conditions (McGanity et al., '54).

[4] Premature infants are those having a birth weight of 2500 gm or less.

the lowering being significant in both the second and third trimesters. The increased frequency of occurrence of premature birth is limited to the group with intakes of vitamin C below 20 mg. Table 7 shows the results for single live-born infants for the third trimester. In the case of serum vitamin C levels, differences are not as large but it appears that the percentage of premature infants is higher for the group with the lowest serum levels. These differences are not necessarily indicative of a causal relation between vitamin C and premature birth since the group with low intakes and serum levels of vitamin C may have been different in other respects from the rest of the study group. The data do suggest an area for further study.

It is of interest to note that mothers of twins had significantly lower serum vitamin C levels in the second and third trimesters, while vitamin C intakes were not significantly different from those of the total study group. Cases of pre-eclampsia had generally lower intakes of all nutrients, including vitamin C, than the total study group. These patterns are believed to be reflections of the illness. There was no tendency for a difference in either vitamin C intakes or serum levels during pregnancy between mothers who later breast-fed their infants and those who did not. As previously noted there were differences in serum levels postpartum. Serum vitamin C levels postpartum tended to be high in those conditions, such as diabetes, where the mother did not nurse her infant.

Lund and Kimble ('43) found no relation of plasma vitamin C levels to the occurrence of toxemia, puerperal morbidity, or duration of labor in 197 cases. They concluded that low values found among cases of hyperemesis probably occurred as a result of the illness. We concur with this position.

It has been reported (Greenblatt, '53; and Javert, '54) that women who were habitual aborters responded favorably to a regime including supplements of vitamin C in the diet along with other measures. In the present study there was no indication of lowered vitamin C nutriture in the 40 women

who aborted. Twenty-three of these had serum vitamin C determinations in the first trimester, and 18 in the second trimester. The distributions did not differ from those for the total study group. Although but a few had diet records, there was no indication of any unusual intake.

Consistent dietary intake groups. As noted earlier, individuals having high (or low) intakes on two or more diet records probably represent a group whose level of intake was generally high (or low). Study of such groups might be expected to show relationships not apparent when classification is based on a single diet record. Consequently, cases having two or more diet records were divided into subgroups as follows:

(1) "consistently low group" — 166 cases having intakes under 40 mg on at least two diet records;
(2) "consistently intermediate group" — 262 cases having intakes of 40 to 80 mg on at least two diet records;
(3) "consistently high group" — 193 cases having intakes of 80 mg or more on at least two diet records:
(4) cases not falling into any of the above groups.

Cases for whom three diet records were available were included in the above groups (1), (2), or (3) if two out of three records satisfied the necessary conditions.

Variation in serum levels of vitamin C for the consistent intake groups. For each of the "consistent" intake groups the median values of serum vitamin C were determined by weeks of gestation in each of two broad seasons, February through June, a period of low blood levels, and July through January, a period of high serum vitamin C levels. Postpartum values were further subdivided according to whether or not the mother was breast-feeding her infant. These are shown in table 8. The small size of the group of cases at 34 weeks gestation and over accounts for the irregularities in this group.

Differences by weeks gestation and weeks postpartum. The group with consistently high intakes maintained essentially

the same average serum vitamin C levels throughout pregnancy but exhibited lowering of the concentrations during the postpartum period.

The group with consistently intermediate intakes showed a decrease in serum concentration between early and late pregnancy. It appears that their intake was sufficient to maintain their average level at about the same value as that of the high intake groups during the first trimester, but was not adequate to maintain these levels throughout pregnancy.

TABLE 8

Median levels of serum vitamin C by weeks gestation and postpartum within consistent intake groups

SEASON	LEVEL OF VITAMIN C INTAKE	WEEKS GESTATION					POSTPARTUM	
		13 and under	14–19	20–26	27–33	34 and over	Not nursing	Nursing
		Median serum vitamin C (*mg/100 ml*)						
Feb.–June	Low	0.18	0.20	0.28	0.14	0.12	0.14	0.12
	Medium	0.54	0.38	0.30	0.34	0.16	0.24	0.12
	High	0.46	0.68	0.58	0.54	0.80	0.36	0.22
July–Jan.	Low	0.40	0.38	0.58	0.30	0.54	0.24	0.16
	Medium	0.52	0.60	0.56	0.40	0.44	0.36	0.18
	High	0.52	0.76	0.56	0.64	0.44	0.48	0.28
		Number of cases						
Feb.–June	Low	24	41	39	71	7	19	20
	Medium	43	52	58	98	20	33	55
	High	28	29	40	85	8	36	43
July–Jan.	Low	32	42	44	77	10	31	55
	Medium	66	75	75	129	11	58	63
	High	41	58	63	85	13	34	42

Values in the postpartum period showed a further decline, significant only for the lactating group.

The group with consistently low intakes had low serum levels early in pregnancy, and a further decrease as pregnancy advanced. Postpartum values were still lower, although in the February-June season 70% of the cases already fell into the lowest grouping interval during the third trimester, so

that there was not much opportunity for observing a further decline.[5]

While differences between third trimester and postpartum values of non-lactating women were not statistically significant within all the individual subgroups, the differences were in the direction of lower values in the postpartum period, and the combined effect of all intake groups in both seasons is significant.

Lactating women had lower serum C levels than non-lactating women in the same intake group, except for those with the lowest intakes where serum C levels of both lactating and non-lactating women were low.

In the consistently high intake group, there were 80 cases who had intakes of 100 mg or over on two or more diet records. During pregnancy their levels were not different from the remainder of the consistently high intake group, but 32 cases who were not nursing their infants at the time of the postpartum laboratory determination had median serum vitamin C levels of 0.70 mg/100 ml. This was significantly higher than the postpartum level of non-lactating women in the remainder of the consistently high intake group. The women who had had intakes of 100 mg or more and who were lactating at the time of the postpartum check-up had median levels of 0.30 mg/100 ml, which is not significantly different from the levels at somewhat lower intakes. Moreover, a group of 18 lactating women whose intakes had been

[5] Differences in median serum C levels between consistent intake groups were not statistically significant in the first trimester except that women with low intakes had lower serum C levels than those with medium and high intakes during the period February through June. In the second trimester, differences were definitely significant in the period February through June, and in the period July through January, those on low intakes had significantly lower serum C levels than those with medium and high intakes. Differences between all intake groups were clearly significant in the third trimester. During the postpartum period differences between intake groups were significant for both lactating and non-lactating women, except that there was little difference between low and medium intake groups among lactating women, both groups having very low serum C levels.

120 mg or more on at least two diet records during pregnancy had median postpartum levels of 0.27 mg/100 ml.

Within each intake group there was considerable individual variation in serum level about the median values, which may be due in part to individual differences in requirements. It is therefore difficult to interpret the results in terms of individual needs, but the figures indicate that on the average a dietary intake level of less than 40 mg was inadequate to support what might be regarded as acceptable concentrations of serum ascorbic acid during pregnancy or lactation. The intermediate daily intake range of 40 to 80 mg did support high levels of serum ascorbic acid early in pregnancy, but failed to do so during the latter part of gestation or during lactation. High serum concentration of vitamin C was supported throughout pregnancy by the consumption of 80 to 100 mg, but during the immediate postpartum period these levels were not attained unless the intake of vitamin C exceeded 100 mg per day. Finally, it appears that intakes in excess of even 120 mg per day did not maintain the average serum level of lactating women above about 0.3 mg.

With the knowledge available at present, it is not possible to say what levels of vitamin C in the serum are optimal, or whether any impairment of health results from lower levels. There is apparently a considerable margin of safety. In deprivation studies (Medical Research Council, '53) about 100 days elapsed between the virtual disappearance of vitamin C from the plasma and the appearance of the first clinical signs of scurvy.

Course and outcome of pregnancy in the consistent low, intermediate and high intake groups. Differences between the three intake groups for each of the following special conditions were within the limits of chance variation: hyperemesis, eclampsia and pre-eclampsia, puerperal fever, stillbirth, neonatal death, birth of single premature [6] infants, and congenital malformation (table 9). There were more cases of

[6] See footnote 4, page 211.

disease conditions [7] in the low intake group, somewhat fewer in the medium intake group, and the smallest number in the high intake group. Low, medium and high groups were also compared for weeks gestation at delivery, labor complications, length of labor, various types of operative deliveries, lacerations occurring at delivery, placenta weight, birth weight of the infant, and type of infant feeding, whether breast or artificial. No significant difference was found. These findings for the "consistent" groups are confirmatory of the analysis

TABLE 9

Occurrence of special conditions in consistent vitamin C intake groups

SPECIAL CONDITIONS	VITAMIN C INTAKE GROUP					
	Low		Medium		High	
	No.	%	*No.*	%	*No.*	%
Disease complicating pregnancy	23	13.9	25	9.5	11	5.7
Hyperemesis	2	1.2	5	1.9	1	0.5
Eclampsia	1	0.6	3	1.1	1	0.5
Pre-eclampsia	12	7.2	14	5.3	9	4.7
Puerperal fever	8	4.8	21	8.0	8	4.1
Stillbirth	1	0.6	3	1.1	0	0
Neonatal death	3	1.8	4	1.5	3	1.6
Live-born premature, excluding twins	8	4.8	5	1.9	8	4.1
Congenital malformations	5	3.0	9	3.4	4	2.1
Total number in group	166		262		193	

of the whole study group (McGanity et al., '54), except for findings relating to premature birth.

Failure to find differences in the number of premature births was surprising in view of the increased incidence of this condition among mothers with intakes under 20 mg, reported above. However, in the present broader classification most of the low group had intakes above the 20 mg level, where no relationship was found. There were 23 women who had intakes of under 20 mg in both the second and third trimesters, and three of these delivered premature infants.

Relation to hematologic values. In view of the lower vitamin C intakes and serum levels among women with anemia

[7] See footnote 3, page 211.

during pregnancy, it is of interest to compare hematologic values for the consistent intake groups, and at the same time to consider associated differences in iron intake.

In the low vitamin C intake group the average hemoglobin, packed cell volume, mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentration, but not the red cell count, were lower than in the other two groups. There was also a difference of about 4 mg in the daily intake of iron between the low and high vitamin C intake groups. In order to judge whether or not differences in hematologic values were attributable to associated differences in iron intake, those women with iron intakes of 10 mg or more in both second and third trimesters were selected within each of the vitamin C intake groups. Mean hematologic values in the third trimester for each of these groups are shown in table 10. Comparison of low and high groups showed significant differences in the second trimester for hemoglobin levels ($P = 0.03$), mean cell hemoglobin ($P = 0.02$) and for mean cell hemoglobin concentration ($P = 0.01$). The only difference which was significant in the third trimester was packed cell volume ($P = 0.05$). No significant differences existed postpartum. We are not able to say whether or not the small observed differences in hematological values are directly associated with vitamin C nutriture. They are of interest in view of the recent reports of Steinkamp, Dubach and Moore ('55) and of Moore ('55) on the effect of ascorbic acid on the absorption of radioiron. Interpretation of our observations is complicated by the possibility that the general abundance of the dietary of women in the high intake category is greater.

Consistently low, intermediate and high serum vitamin C groups. Cases were divided into low, medium and high serum vitamin C groups on the basis of second and third trimester determinations:

(1) "consistently low group" — 442 cases having serum vitamin C levels under 0.40 mg/100 ml in both the second and third trimesters or between 0.40 and 0.60 mg/100 ml in the second trimester and under

TABLE 10

Hematologic values in consistent vitamin C intake groups and in consistent serum vitamin C groups, third trimester

(Cases with iron intakes of 10 mg/day or more second and third trimesters)

CONSISTENT GROUP	NO. OF CASES	HEMOGLOBIN	RED BLOOD COUNT	PACKED CELL VOLUME	MEAN CELL VOLUME	MEAN CELL HEMOGLOBIN	MEAN CELL HEMOGLOBIN CONCENTRATION
		gm/100 ml	*million/mm³*	%	μ^3	$\mu\mu g$	%
Vitamin C intake							
Low	67	11.44 ± 0.16	3.89 ± 0.05	34.6 ± 0.4	88.6 ± 1.0	29.24 ± 0.41	32.94 ± 0.25
Medium	199	11.55 ± 0.10	3.92 ± 0.03	35.0 ± 0.2	90.2 ± 0.6	29.76 ± 0.24	33.06 ± 0.14
High	164	11.72 ± 0.10	3.93 ± 0.03	35.5 ± 0.3	90.5 ± 0.7	30.02 ± 0.26	33.04 ± 0.16
Serum vitamin C							
Low	242	11.44 ± 0.09	3.92 ± 0.03	34.6 ± 0.2	88.8 ± 0.5	29.32 ± 0.22	32.98 ± 0.13
Medium	139	11.61 ± 0.11	3.96 ± 0.04	35.0 ± 0.3	89.3 ± 0.7	29.54 ± 0.29	33.08 ± 0.17
High	128	11.69 ± 0.12	3.87 ± 0.04	35.3 ± 0.3	91.4 ± 0.7	30.40 ± 0.30	33.12 ± 0.18

0.20 mg/100 ml in the third trimester; those having serum levels under 0.20 mg/100 ml in both the second and third trimesters were later separated out from this "low" group and designated as the "very low" group (162 cases);

(2) "consistently intermediate group" — 249 cases having serum levels of vitamin C between 0.40 and 0.80 mg/100 ml in the second trimester and between 0.20 and 0.80 mg/100 ml in the third trimester;

(3) "consistently high group" — 198 cases with serum vitamin C levels of 0.80 mg/100 ml or more in either second or third trimester and 0.60 mg/100 ml or more in the other trimester.

The occurrence of various special conditions within the three groups is shown in table 11. The following conditions did not differ between the groups: hyperemesis, eclampsia and pre-eclampsia, stillbirth, neonatal death or congenital

TABLE 11

Occurrence of special conditions in consistent serum vitamin C groups

SPECIAL CONDITIONS	CONSISTENT SERUM C GROUPS									
	Very low		Medium low		Total low		Medium		High	
	No.	%	*No.*	%	*No.*	%	*No.*	%	*No.*	%
Disease complicating pregnancy	23	14.2	29	10.4	52	11.8	30	12.0	12	6.1
Hyperemesis	3	1.9	2	0.7	5	1.1	2	0.8	3	1.5
Eclampsia	2	1.2	1	0.4	3	0.7	3	1.2	1	0.5
Pre-eclampsia	10	6.2	17	6.1	27	6.1	12	4.8	6	3.0
Premature separation of placenta	2	1.2	7	2.5	9	2.0	1	0.4	0	0
Puerperal fever	6	3.7	23	8.2	29	6.6	15	6.0	5	2.5
Stillbirth	2	1.2	3	1.1	5	1.1	5	2.0	1	0.5
Neonatal death	3	1.9	7	2.5	10	2.3	2	0.8	4	2.0
Live-born premature, excluding twins	13	8.0	9	3.2	22	5.0	10	4.0	4	2.0
Congenital malformations	1	0.6	14	5.0	15	3.4	2	0.8	7	3.5
Total number in group	162		280		442		249		198	

malformation. Puerperal fever was significantly lower in the group with highest serum levels, allowance being made for differences by season and parity in both serum vitamin C and in the occurrence of puerperal fever. No relation of puerperal fever to intake of vitamin C was observed, nor was any relation to serum levels apparent when data for single trimesters were studied. We conclude that ascorbic acid nutriture is not a major factor in the occurrence of puerperal fever.

Differences in per cent of premature [8] single births and disease conditions complicating pregnancy [9] were not statistically significant on the basis of the initial division of cases; however, when the low group was further subdivided so as to separate out 162 cases having values of less than 0.20 mg/100 ml in both trimesters, both the percentage of cases of premature single live births and percentage of cases having complicating diseases were significantly higher in the low group. The disease conditions showing higher than average frequency in this group were (a) venereal disease or a history of syphilis (9 cases), and (b) pyelitis, cystitis, pyelonephritis, and pyonephrosis (5 cases).

Among labor complications there was a significant difference for premature separation of the placenta (table 11). Serum vitamin C levels were not related to the gestation time at delivery. However, within the group of women who gave birth to premature infants (on the basis of birth weight) there was a tendency for the cases in the group with low serum vitamin C to have shorter gestations. Sixteen of 22 single live-born prematures in the low group had gestations of less than 38 weeks. For the medium group the corresponding numbers were 5 of 10, and for the high group none of 4. (Premature single live-birth was associated with premature separation of the placenta in only two cases.) Consistent serum vitamin C groups were also compared for length of labor, various types of operative deliveries, lacerations oc-

[8] See footnote 4, page 211.
[9] See footnote 3, page 211.

curring at delivery, placenta weight, birth weight of the infant, and type of infant feeding. No significant difference was found.

Hematologic values varied with serum vitamin C levels in a manner similar to that discussed above for intake values. Comparison of low and high groups (with iron intake of 10 mg or more in both second and third trimesters) showed significant differences for packed cell volume in all trimesters, and for hemoglobin, mean cell volume and mean cell hemoglobin in the third trimester (table 10 shows data for the third trimester).

In evaluating these results it should be noted that a causal relationship cannot be established from data of this type. Groups of cases selected on the basis of one characteristic, e.g. serum levels of vitamin C, will be expected to differ in other characteristics. In interpreting the data, we have allowed for differences recognized to exist, such as those for parity or season of the year, but it is obvious that other differences may be present. Such possibilities cannot be ruled out in an observational study.

SUMMARY

Data on intakes and serum levels of vitamin C in 2,129 pregnant women are studied in relation to many factors, including the course and outcome of pregnancy.

In general, serum levels decreased during pregnancy except in the group at a high level of intake. Values were further decreased postpartum, and were lower for lactating than for non-lactating women. Evidence is presented that on the average intakes of 80 to 100 mg daily supported high levels of ascorbic acid in the serum during pregnancy. The serum levels of non-lactating mothers averaged 0.7 mg per 100 ml during the puerperium on intakes (during pregnancy) of 100 mg or over per day; the serum concentration of lactating mothers did not average greater than 0.3 mg even on intakes exceeding 120 mg daily.

Analysis of findings relative to the health of the mother and baby revealed only 5 categories which may possibly be associated with ascorbic acid nutriture: hematologic findings, gingivitis, premature separation of the placenta, premature birth, and puerperal fever. Increased frequency of premature birth was limited to the lowest intake levels and lowest serum concentrations. In none of the conditions was there a strong relation to both intakes and serum levels. Hence we believe that ascorbic acid nutriture is at most a contributary factor in any of these.

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NUTRITION OF SALMONOID FISHES

III. WATER-SOLUBLE VITAMIN REQUIREMENTS OF CHINOOK SALMON [1]

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INTRODUCTION

Early attempts to investigate the vitamin requirements of fish were hampered by the lack of a vitamin-test diet in which the growth factors could be adequately controlled. McCay and Dilley ('27) postulated the requirement of trout for a factor H present in fresh meats. General requirements for certain vitamins were recognized (McCay, Bing and Dilley, '27; Titcomb et al., '29; Tunison et al., '42; Wolf, '42; Woodbury, '42) and under certain conditions anemia could be prevented by the administration of xanthopterin (Simmons and Norris, '41) or some B vitamin combinations (Tunison et al., '43). McLaren et al. ('47b) published a diet consisting of commercial casein, starch, fish liver oils, crab meal, minerals and vitamins which would maintain rainbow trout *(Salmo gairdneri)* for a growing period of 20 weeks. Feeding trials with chinook salmon *(Oncorhynchus tshawytscha)* and extended feeding trials with this diet and rainbow trout were unsatisfactory (Rucker, Johnson and Kaydas, '52). Wolf ('51) observed some vitamin deficiency syndromes in rainbow trout fed a test diet of commercial casein, potato starch, gelatin, minerals, hydrogenated cottonseed oil, cod liver oil, and crystalline vitamins. The requirements of rainbow trout

[1] Presented at the meetings of the American Institute of Nutrition, Atlantic City, New Jersey, April, 1956.

for pantothenic acid, inositol, and folic acid were reported but the niacin, biotin, ascorbic acid, vitamin B_{12}, alpha-tocopherol and vitamin K-deficient lots of fish failed to develop observable deficiency syndromes (Wolf, '51). Using vitamin-test casein, previously reported unsatisfactory for trout feeding studies (Wolf, '51), changing the fat and carbohydrate components, and slightly altering the mineral and crystalline vitamin supplements, a vitamin-test diet was formulated for feeding trials with chinook salmon as the experimental animal.

EXPERIMENTAL

Experiment 1. Complete diets. The complete vitamin-test diet was formulated from the materials listed in table 1. Prior to mixing the diet, the crystalline vitamin supplement, the amino acid supplement and the alpha-cellulose flour were mixed for two hours in a ball mill and then stored at 5 to 10° C., until used. To insure more accurate and reproducible weights, sufficient alpha-cellulose flour, amino acids and vitamins for at least 4 kg of diet were mixed at one time. The mineral mixture was also ground for two hours in a ball mill and stored in a tight container until needed.

To prepare 400 gm of mixed diet containing 25% solids and 75% water, 15 gm of purified gelatin were added to 300 ml of water and heated on a steam bath until the temperature rose to 60 to 70°C. After the gelatin became liquefied, the container was removed from the steam bath, placed in a mechanical mixer and stirred at medium speed with a dough hook until the temperature had dropped to 40 to 50°C. Then 54 gm vitamin test casein, 9 gm purified corn oil, 8 gm white dextrin and 4 gm mineral mixture were added and thoroughly blended. Finally, the alpha-cellulose flour containing the vitamin mixture and the amino acid supplement was added and stirred until a homogeneous mass was obtained (30 to 35°C.). For convenience in future feeding, the mixture was poured into ice cube containers, hardened in a refrigerator at 10°C., and stored in screw-top glass jars at 5 to 10°C. until used.

TABLE 1

Composition of the vitamin-test diet for chinook salmon

MAIN MIXTURE	PARTS	MINERAL MIXTURE	AMOUNT	VITAMIN SUPPLEMENT	AMOUNT [1]
					mg
Vitamin-free casein	54	U.S.P. XII Salt Mixture No. 2	100 gm	Thiamine-hydrochloride	6
Gelatin, purified	15	Aluminum chloride	18 mg	Riboflavin	20
Corn oil, purified	9	Zinc sulfate	357 mg	Pyridoxine hydrochloride	4
White dextrin	8	Cuprous chloride	11 mg	Nicotinic acid	80
Alpha-cellulose flour	9	Manganous sulfate	80 mg	Calcium pantothenate	28
Mineral mixture	4	Potassium iodide	17 mg	Inositol	400
DL-Methionine	1	Colabtous chloride	105 mg	Biotin	0.6
L-Tryptophan	0.5			Folic acid	1.5
Crystalline vitamin supplement				p-Aminobenzoic acid	40
				Choline chloride	800
				Ascorbic acid	200
				Alpha-tocopherol	40
				Menadione	4
				Beta-carotene	1.2
				Activated 7-dehydrocholesterol	0.0045
				Crystalline vitamin B_{12} [2]	0.009

[1] These amounts of individual vitamins were added per 100 gm of the main mixture.

[2] Merck.

The resulting diet was a firm homogeneous mass which could be fed easily through a food ricer, sieve or grater. Correct particle size was adjusted by the size of the grater or ricer used in feeding, and as the fish developed, the consistency of the diet was altered to 30% solids and 70% water. Similarly, the density of the diet was varied from a floating diet for initial feeding to a slowly sinking diet for larger fish by adjusting the speed of the mechanical mixer during the cooling and mixing period.

Four groups of 1,000 yolk-sac chinook salmon fry, a representative random sample from one day's egg take at the State of Washington Department of Fisheries' Soos Creek Hatchery, were placed in screened troughs supplied with dechlorinated Seattle city water and taught to feed on finely ground fresh beef liver. After three weeks, all the yolk-sacs had been absorbed and the lots of fish were feeding actively on the liver diet. Two 1,000 fish lots were then taught to feed on the complete vitamin-test diet and the other two were continued on beef liver. In general, the techniques used in the hatchery followed closely the techniques developed by the Fish and Wildlife Service for conducting feeding trials (Burrows, Palmer and Robinson, '51). Bi-monthly growth measurements, daily mortalities and daily food consumption were recorded on all groups for a feeding period of 18 weeks.

After the fry were actively feeding, only as much diet as the fish would eat completely within one minute after presentation was grated into the upper one-third of each trough. The fish were fed three times daily, 6 days per week, with no diet fed on Sundays. As the fish developed in size, the water and air content of the ration was reduced, and after the fish were an average size of one gram, a slowly sinking diet containing 30% solids and 70% water was prepared twice weekly for the remainder of the experiment. After 6 weeks of feeding, all lots were reduced to 500 fish per trough to prevent overcrowding. Freshly ground beef liver, obtained from the School of Fisheries production diet, was fed through a ricer or sieve to the paired control lots at the same level

and frequency as the test diet lots. The growth-mortality curves and the food consumption were plotted as shown in figure 1 and table 2.

Experiment 2. Vitamin-deficient diets. The following year, a different group of chinook salmon fry from a different natural water supply was used to check the adequacy of the complete test diet on other runs of chinook salmon and to develop specific vitamin deficiency syndromes by deleting one water-soluble vitamin at a time from the complete vitamin mixture in the diet. Consequently 13,000 fry from three summer-run chinook salmon females were obtained from the

TABLE 2

Growth, mortality and food consumption of chinook salmon

RATION	NO. FISH	INITIAL WEIGHT	FINAL WEIGHT	PER CENT GAIN	PER CENT MORTALITY [1]	FOOD FED [2]	FOOD VERSION CONRATIO	WEEKS OF FEEDING
		gm	*gm*			*gm*		
Test diet	1000	0.49	5.31	984	5.58	11080	4.3	18
Test diet	1000	0.49	5.49	1020	8.37	11080	4.5	18
Liver	1000	0.49	6.97	1322	5.87	19190	4.0	18
Liver	1000	0.49	6.77	1280	7.07	19190	4.3	18

[1] Mortality not attributable to Columnaris disease.

[2] Calculated on the basis of 25% solids diet.

U. S. Fish and Wildlife Service Entiat River Salmon Cultural Station, transported to the laboratory in a tank truck, acclimated to the experimental hatchery water supply and taught to feed on the complete test diet. Since these fry were actively feeding when obtained and had been on a production diet containing large amounts of liver and other vitamin rich animal tissues, it was decided to hold the fry on the test diet without the vitamin supplement at least three weeks before initiating the deficiency diet tests in an attempt to decrease as much as possible the stored biological materials in their systems. After three weeks the entire lot was weighed, combined in one trough, crowded into the head end of the trough, and representative random samples of 500 fish were

hand counted and weighed to within one gram on a solution balance. Each group of 500 fry weighed 745 ± 2 gm, substantiating the accuracy of the sampling technique used.

Throughout the remainder of the experiment, all weighings were conducted by one individual (JEH) and, by periodically

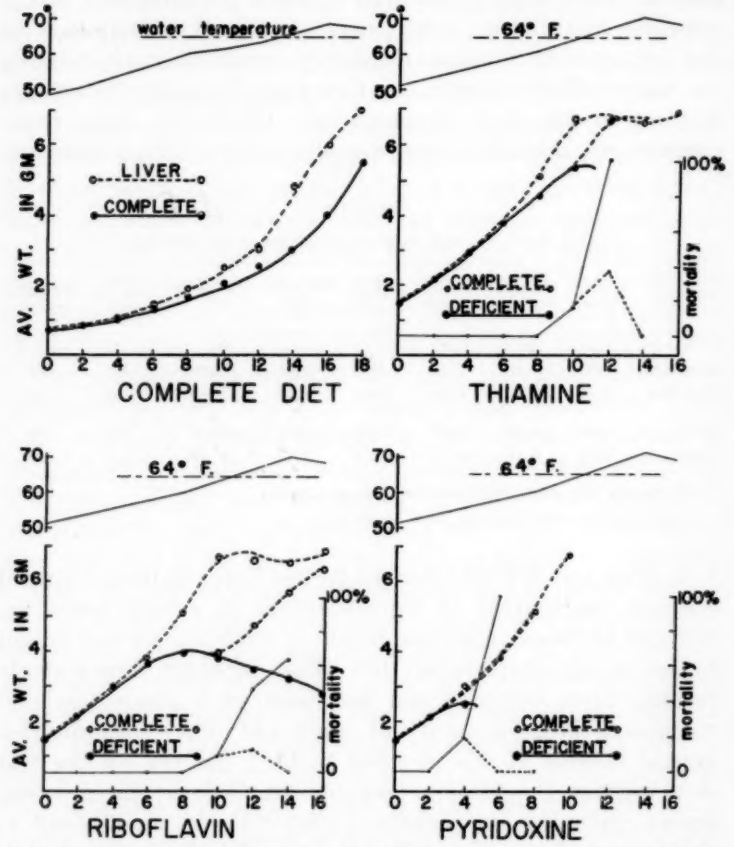

Fig. 1 Growth and mortality of chinook salmon fed complete diets and diets deficient in thiamine, riboflavin or pyridoxine. *Upper* curves, average water temperature; *central* curves, growth; *lower* curves, bi-weekly mortality percentage of the deficient group. Junction points in growth and mortality curves represent division of the deficient groups into two sublots after the deficiency syndromes became apparent in a large portion of the population.

conducting a series of weights on each group, were shown to be reproducible to within 1%. The entire population of each trough was weighed prior to the morning feeding by removing the entire population in one net, draining off the excess water over a 10 second period, wiping the bottom of the net clear of adhering water and transferring the fish to a tared container with sufficient water to maintain the fish during the weighing period. After weighing, the fish were immediately returned to their trough and in one-half hour were fed.

Individual brushes and food graters were marked at each trough to minimize transfer of nutrients or pathogens when cleaning or feeding. Each diet jar was numbered and labelled to correspond with the number and label on each trough to prevent inadvertent erroneous feeding. All troughs were cleaned daily to prevent the accumulation of feces and the growth of bacterial and fungus matter. Food was grated into the upper one-third of each hatchery trough three times daily throughout the experimental feeding period. Only as much food was fed as the fish would eat within one minute after presentation.

Daily mortalities were removed and recorded as soon as observed. Dead or obviously moribund fish were examined closely for the presence of fish pathogens and for indications of the various vitamin deficiency syndromes listed by McLaren et al. ('47a), Tunison et al. ('44), and Phillips et al. ('45, '47, '49, '50) for trout.

Since only 14 experimental feeding troughs were available in the hatchery for these vitamin deficiency studies, the experiments could not be conducted in duplicate. This disadvantage was not serious, however, in view of the feeding results and the techniques used. It was obviously significant when over half the population died exhibiting various specific vitamin deficiency syndromes and the other portion of the population in the trough recovered after replacement of the missing vitamin in the diet. For further evidence on the significance of the mortalities, the mortality percentage of

each group was examined by the Chi-square Test. Similarly, the difference in growth rates between the controls and those on deficient diets were examined for significance at the 95% confidence level by the Student t_N Test at the end of 10, 12, and 14 weeks of feeding respectively.

The general approach to developing the various vitamin deficiencies included feeding each group of fish in the 12 troughs one water-soluble vitamin-deficient diet and allowing the deficiency syndromes to develop until 20% of the original population of 500 fish had died. The remaining population was divided in half, weighed to confirm random division, the lower one-half of the trough fed the complete vitamin-test diet and the upper one-half allowed to continue on the deficient diet until complete elimination of that population. For purposes of comparison, two troughs were fed the complete vitamin-test diet throughout the experimental-feeding period as controls. Two parts of cod liver oil were substituted for two parts corn oil and the crystalline beta-carotene and activated 7-dehydrocholesterol were deleted from the crystalline vitamin supplement for the deficiency-feeding trials.

Assays of the levels of residual vitamin activity present in the vitamin-deficient diets were completed on the solid ingredients of the diet and the diet as fed (25% solids and 75% water). Duplicate samples were assayed at 5 levels and compared with standards in triplicate at 10 levels in conformance with standard microbiological assay techniques. All organisms used in the microbiological assays were fresh cultures obtained directly from the American Type Culture Collections, Washington, D. C. Thiamine was run by the method of Sarett and Cheldelin ('44) using *L. fermenti* 36; riboflavin, nicotinic acid and folic acid by the method of Roberts and Snell ('46) using *S. faecalis* R; pantothenic acid by the hydrolysis method of Novelli and Schmetz ('51) and the assay method of Skeggs and Wright ('44) using *L. arabinosis* 17–5; inositol by the method of Sawyer ('51) using *Kloeckera brevis;* biotin by the method of Wright and Skeggs ('44) using *L. arabinosis* 17–5; para-aminobenzoic acid

by the method of Lewis ('42) using *L. arabinosis* 17–5; and choline by the method of Horowitz and Beadle ('43) using *Neurospora crassa* "cholineless." Vitamin B_{12} was assayed by Miss Neva Kerrick in the microbiological assay laboratories of the Fish and Wildlife Service Branch of Commercial Fisheries in Seattle by the method of Hoffman et al. ('49) using *L. leichmannii.* Ascorbic acid was determined by the 2–4 dinitrophenylhydrazine method of Roe and Oesterling ('44). Details of the results were tabulated in table 3.

Experiment 3. Deficient diets at constant temperatures. Experiment 2 was repeated with another lot of fish at a different laboratory using a constant temperature water supply (58°F.). The diets deficient in one water-soluble vitamin were fed to paired lots of salmon fry from a third source of fish (Columbia River fall run chinook salmon) at the Hagerman, Idaho Nutrition Laboratory. Feeding techniques and biological observations during the 20-week feeding period were identical with the previous experiment.

Since these groups of deficient fish were used as negative control lots in a quantitative experiment,[2] replacing the missing vitamin for recovery experiments in depleted groups was not possible. Therefore, these groups were continued beyond the point where the majority of the population died from deficiency syndromes.

RESULTS AND DISCUSSION

Experiment 1. After 16 weeks on the experimental diets, the water temperature rose to above 65°F. and a severe epizootic of Columnaris *(Chondrococcus columnaris)* occurred, forcing termination of the feeding trials at the end of the 18-week period. At the end of the experiment, total blood cell counts, hemoglobin determinations, microscopic examinations of gill filaments and post-mortem examinations of the liver, spleen, kidney, stomach, pyloric caeca and intestines were made on 20 fish from each lot. No abnormalities in mortality,

[2] Details of quantitative experiments to be published later.

food consumption, total blood cell counts, hemoglobin determinations, gill filament examinations, or post-mortem examinations of the internal and external organs were observed in any fish from any of the 4 experimental lots. During the last two weeks of the experiment, all dead fish were examined for Columnaris and in each experimental lot groups of *Chrondococcus columnaris* were observed. Particular emphasis was placed on observing any of the vitamin deficiency syndromes reported by McLaren et al., ('47b) in rainbow trout, but by the end of the experiment no recognizable vitamin deficiency syndrome could be detected in any salmon from either of the paired groups of fish.

During the entire feeding period the lots of fish on both the liver and the complete vitamin-test diet exhibited a healthy appetite and would consume all food offered within the feeding period. No indications of food refusal were noted, and as long as the ration presented did not exceed the appetite, little wastage was observed. Some of the nutrients were obviously lost due to leaching in the water before the salmon could consume them, but this effect was not excessive in view of the food consumption and growth results summarized in table 2.

Since the epizootic of Columnaris disease prevented accurate calculations of growth rates, food consumption and normal mortalities during the last two weeks of the feeding trials, the experiment was terminated after 18 weeks of feeding. Even though the growth rates indicated that the fish on the test diet would approach or exceed that of the groups on the liver diet within 20 to 22 weeks, it was doubtful whether these fish could have been held in troughs for an additional two weeks because of the "space factor" effect described by Johnson and Gastineau ('52) or because of the migratory urge which appears in groups of chinook salmon held in troughs when the average weights of the individual fish exceed 7 to 9 gm.

The differences of the means of each group of points forming the two growth curves in figure 1 were examined by the

Student t_N Test and were found acceptable except those points representing the 14th and 16th weeks of feeding. These two sets of points were rejected by this test and must therefore be regarded as significant differences at the 95% confidence level.

Throughout the experimental feeding period the experimental animals gave every indication of being healthy. None of the tests indicated that they were abnormal in any respect.

TABLE 3

Vitamin assays of deficient diets for chinook salmon [1]

DEFICIENT DIET	SAMPLE	VITAMIN MIX	DIET [2]	DETECTABLE	METHOD USED
	gm	*μg/gm*	*μg/gm*	*μg/gm*	
Thiamine	10	0.25	0.1	0.01	MBA [3]
Riboflavin	10	2.5	0.22	0.1	MBA
Pyridoxine	10	NDA [4]	0.07	0.01	MBA
Nicotinic acid	10	0.6	0.7	0.1	MBA
Pantothenic	10	1.9	0.24	0.1	MBA
Inositol	10	20.0	17.0	5.0	MBA
Biotin	10	0.0165	0.0053	0.002	MBA
Folic acid	10	0.75	5.9	0.5	MBA
p-Aminobenzoic	10	NDA	NDA	0.1	MBA
Choline	10	7.0	4.5	1.0	MBA
Ascorbic acid	100	3.5	1.0	0.5	Chem
Vitamin B_{12}	10	NDA	0.005	0.0001	MBA

[1] Mean values reported of duplicate samples each of vitamin mixture and diet.
[2] Calculated on basis of diet containing 25% solids and 75% water.
[3] Microbiological assay method.
[4] No detectable amount.

Although the growth rates for the fish on the test diets were below those on the liver diet, all examinations revealed healthy fish without the appearance of any recognizable vitamin deficiency syndrome. Total blood cell counts made by caudal peduncle amputation, blotting, dilution in red blood cell hemacytometers with gentian violet in saline solution, and counting under a microscope revealed a normal blood cell count of all fish on all diets between 1,250,000 and 1,500,000 rbc/mm^3. Hemoglobin determinations made on Talquist-Adams paper varied between 10.9 and 14.1 gm/100 ml blood for all fish

examined. Microscopic examinations of gill filaments and post mortem examinations of livers, spleen, kidneys, stomachs, pyloric caeca and intestines showed the absence of any recognizable pathological condition. The abdominal fat was slightly excessive in those fish on the liver diet, but this was not considered significant in view of the reported characteristics of body fat in fish (Norris and Donaldson, '40). With the exception of the Columnaris infection which grew to epizootic proportions during the last two weeks on the diet, no pathological condition or deficiency syndrome could be defined.

Experiment 2. The deficiency syndromes observed and the growth curves of each vitamin-deficient lot of salmon are shown in table 4 and figures 1–3.

An apparent decrease in dietary intake was observed in the 12th week of the experiment when the water temperature rose to over 65°F. Near the end of the feeding period, the temperature decreased with a consequent increase in appetite and growth in the lots of fish fed the complete diet. A more constant temperature of the water supply within the range of 50 to 60°F. probably would have strengthened the interpretation of the feeding trials.

Since tryptophan was included in the supplement to the protein component, it was to be expected that the nicotinic acid-deficient group of fish would be delayed in exhibiting the deficiency syndrome. Fortunately, after 13 weeks of feeding, the mortality in this lot rose to 20% of the original population and the group could be split. During the last three weeks of feeding the deficiency syndromes became more apparent and the mortality continued to increase in the deficient lot. Continuing this experiment further probably could have shown a greater divergence between the recovery and deficient groups.

The choline-deficient group exhibited little mortality until the 14th week of feeding, but the growth of the fish was very slow. Probably the methionine content of the diet acted as a partial methyl donor and tended to spare the requirement for choline.

TABLE 4

Signs of avitaminosis in chinook salmon fed vitamin-deficient diets

VITAMIN DEFICIENCY	CHINOOK SALMON	OTHER ANIMALS [1,2]
Thiamine	Poor appetite; muscle atrophy; convulsions prior to death; instability and loss of equilibrium.	Poor appetite; muscle atrophy; convulsions in most acute stage; generalized edema; instability and loss of equilibrium; brain lesions; degeneration of peripheral nerve fibers; impaired carbohydrate metabolism.
Riboflavin	Corneal vascularization; cloudy lens, hemorrhagic eyes; photophobia; dim vision; incoordination; abnormal pigmentation of iris; striated constrictions of abdominal wall; dark coloration; poor appetite.	Corneal vascularization; cloudy lens; hemorrhagic eyes; cataracts photophobia; dim vision; incoordination; abnormal pigmentation of iris; scleral congestion; cheilosis and angular stomatitis; impaired erythrocyte formation.
Pyridoxine	Nervous disorders; epileptiform fits; hyperirritability; ataxia; anemia; loss of appetite; edema of peritoneal cavity; colorless serous fluid; blue-green coloration on back; post-mortem rigor mortis occurs rapidly; rapid and gasping breathing; flexing of opercles.	Nervous disorders; epileptiform fits; hyperirritability; ataxia; anemia; loss of appetite; edema; symmetrical dermatosis; dermal lesions; iron deposition in liver; rise in serum iron.
Pantothenic acid	Clubbed gills; prostration; loss of appetite; necrosis and scarring; cellular atrophy; gills covered with exudate; sluggishness; general "mumpy" appearance.	Prostration; loss of appetite; necrosis and scarring; cellular atrophy; spectacle alopecia; generalized scaling and dermatitis; diarrhea; hyperemia of intestine; myelin degeneration of nerves.
Inositol	Poor growth; distended stomach; increased gastric emptying time.	Poor growth; increased gastric emptying time; alopecia; nutritional encephalomalacia.
Biotin	Loss of appetite; lesions in colon; coloration; muscle atrophy; spastic convulsions; fragmentation of erythrocytes.	Loss of appetite; poor growth; muscle atrophy; seborrheic skin disease; generalized erythema; spastic gait; necrosis of fibers; increased sarcolemma.
Folic acid	Poor growth; anemia; lethargy; fragility of caudal fin; dark coloration.	Poor growth; lethargy intersposed with convulsions; megaloblastic erythropoesis; nutritional cytomenia; infarction of spleen.
Choline	Poor growth; poor food conversion; hemorrhagic kidney and intestine.	Poor growth; poor food conversion; aversion to food; fatty infiltration of liver; necrosis and scarring; reddish kidney; hemorrhagic eyes.
Nicotinic acid	Loss of appetite; lesions in colon; jerky or difficult motion; weakness; edema of stomach and colon; muscle spasms while apparently resting.	Loss of appetite; lesions in colon; jerky or spastic gait; weakness; pain; skin eruptions; edema of stomach and colon; anorexia; diarrhea; neurological lesions; fatty livers; vessel dilation.
Vitamin B_{12}	Poor appetite; erratic hemoglobin and erythrocyte counts; fragmentation.	Pernicious anemia; macrocytic anemia; sprue; low or poor growth in young.
p-Aminobenzoic acid	No abnormal indication in growth, appetite, mortality.	Alopecia in rat and poor growth in chick.
Ascorbic acid	No abnormal indication in growth, appetite, mortality.	Scurvy; capillary fragility; immature fibroblastic formation.

[1] West and Todd ('51).
[2] Wooster ('54).

Under the experimental conditions used, inconclusive results were obtained with the vitamin B_{12}-deficient group of fish. No abnormal indications in appetite, growth, mortality or post-mortem examinations of external and internal organs were observed in the ascorbic acid- or para-aminobenzoic acid-deficient lots of salmon. The average weight of these fish

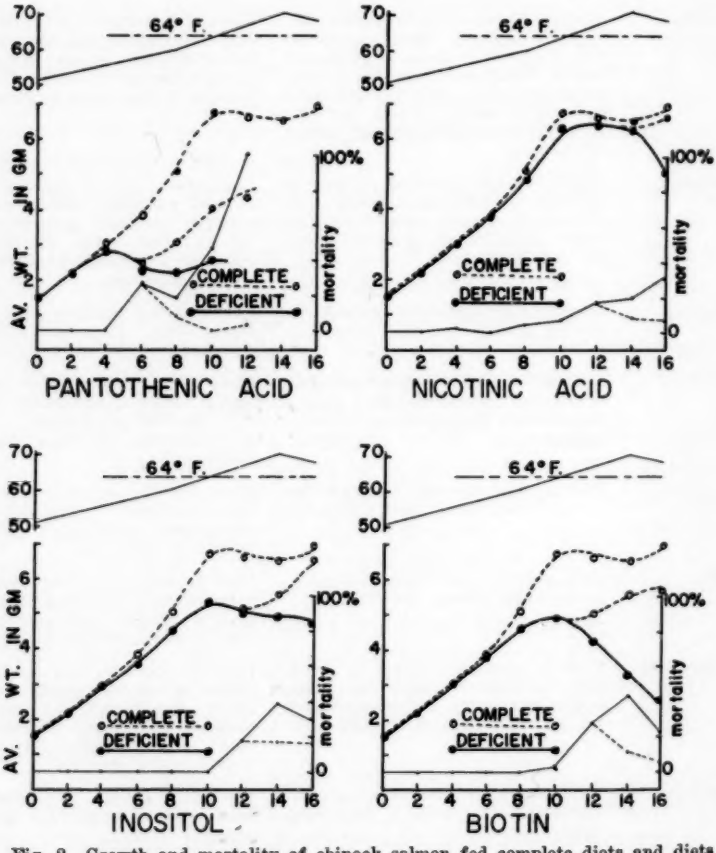

Fig. 2 Growth and mortality of chinook salmon fed complete diets and diets deficient in pantothenic acid, nicotinic acid, inositol or biotin. For identification of various curves, see legend for figure 1.

paralleled closely those of the complete test lots and a simple Student t_N test did not differentiate between any specific point on the curves comparing growth of the control lots with vitamin B_{12}-, ascorbic acid-, or para-aminobenzoic acid-deficient groups of salmon.

Individual samples from each of the deficient groups of fish were removed for histological analysis immediately following the bi-weekly weighing period. Total blood cell counts

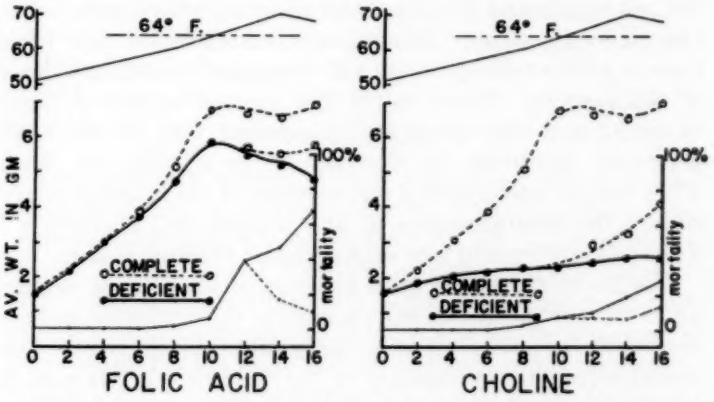

Fig. 3 Growth and mortality of chinook salmon fed complete diets and diets deficient in folic acid or choline. For identification of various curves, see legend for figure 1.

and hemoglobin determinations of these samples seemed normal in all lots of fish with the exception of folic acid- and vitamin B_{12}-deficient groups. A large number of undeveloped nuclei and low total blood cell counts (1,000,000 to 1,100,000) were observed in the folic acid-deficient lot and the hemoglobin determinations averaged 9.0 gm/100 ml blood. In the vitamin B_{12}-deficient group, erratic hemoglobin values were found varying from 9.0 to 13.5 gm/100 ml blood and the total blood cell counts varied from 900,000 to 1,500,000. In the larger fish of this group the erythrocytes appeared wrinkled or fragmented and many undeveloped cells were present.

Unfortunately a hematologist was not available for complete study of these blood cells.

When the missing vitamin was returned to the deficient groups of fish, recovery was rapid in most cases, with the disappearance of observable deficiency syndromes. In the case of riboflavin-deficient fish where the eye damage had resulted in complete blindness, these individual fish failed to eat and soon died. When only one eye was badly affected the fish did recover normal body functions, but the damaged eye did not repair and had no detectable vision at the end of the experimental period. Pantothenic acid-deficient fish varied from a mild swelling of the gill filaments to a severe flaring of the opercles. These latter fish seemed to have difficulty in eating and only about 50% recovered with no observable deficiency syndrome by the end of the experiment. In all other lots of deficient fish, replacement of the missing vitamin caused the disappearance of any observable deficiency syndrome by the end of the experimental feeding period.

Results of the histological analysis of the normal, deficient and recovery groups of fish on each of these vitamin deficient diets will be published at a later date. At that time interpretation of the reversibility of the deficiency syndromes will be discussed.

Experiment 3. Using a water supply with a constant temperature (58°F.) and paired troughs of 300 fish each, confirmatory specific deficiency syndromes were observed with diets deficient in thiamine, riboflavin, pyridoxine, biotin, pantothenic acid, folic acid, inositol, nicotinic acid, or choline. Vitamin B_{12}-deficient fish again failed to show conclusive symptoms.

SUMMARY

A vitamin-test diet consisting of a vitamin-free basal ration supplemented with crystalline vitamins was formulated which would maintain chinook salmon fingerlings for a period sufficient for the development of water-soluble vitamin deficiency syndromes.

Deleting the water-soluble vitamins one at a time from the complete test diet caused typical specific deficiency syndromes in chinook salmon which were comparable to those observed in other experimental animals.

Requirements for thiamine, riboflavin, pyridoxine, pantothenic acid, niacin, inositol, biotin, folic acid and choline were demonstrated.

Inconclusive results were obtained for vitamin B_{12}-deficient fish.

Under the experimental conditions used, no deficiency syndromes were observed in ascorbic acid- or para-aminobenzoic acid-deficient lots of salmon.

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NUTRITION OF SALMONOID FISHES

IV. AN AMINO ACID TEST DIET FOR CHINOOK SALMON [1]

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INTRODUCTION

A prerequisite for determining the amino acid requirements of salmon should be the development of a satisfactory test diet which will maintain chinook salmon *(Oncorhynchus tshawytscha)* for a period sufficient for the development of any amino acid deficiency syndrome. In order to allow the investigator the greatest experimental control in adjusting the levels of individual amino acids in the protein component of the diet, a mixture of crystalline amino acids was desirable for the nitrogen source. Adequate levels of vitamins, carbohydrates, fats and minerals for at least a 20-week feeding period with chinook salmon fingerlings were established previously (Halver, '57). Wood, Griffin and Snieszko ('54) tested various synthetic inert binders for fish diets and found that carboxymethylcellulose (CMC) was suitable for used in studies with purified diets. Combining the above ingredients and furnishing the nitrogen source with natural L-isomers of the amino acids common to plant and animal proteins, an amino acid test diet was formulated and tested against the complete vitamin-test diet previously mentioned (Halver, '57).

EXPERIMENTAL

As a first approximation of the levels of individual amino acids to be fed as the nitrogen source, keeping the other major

[1] Presented at the meetings of the American Institute of Nutrition, Atlantic City, New Jersey, April, 1956.

components of the ration as close as possible to those used previously in the vitamin-test diet, assays were completed on the amino acid composition of yolk-sac fry and initial-feeding fingerlings. These values differed from the calculated levels of amino acids in the casein-gelatin protein component of the control diet; it seemed appropriate, therefore, to test

TABLE 1

Amino acid composition of yolk-sac fry and salmon fingerlings and of experimental diets

INGREDIENT	MICROBIOLOGICAL ASSAYS			DIET MIXTURES	
	Yolk-sac fry	Salmon fingerlings	Control	C-G	YSF
	gm	*gm*	*gm*	*gm*	*gm*
L-Arginine(· HCl)	1.41	1.36	4.44	5.00	2.50
L-Histidine(· HCl · H_2O)	2.07	1.80	2.13	2.50	3.00
L-Isoleucine	4.76	5.38	4.11	4.00	6.00
L-Lysine(· HCl)	2.09	2.07	5.05	5.00	3.00
L-Methionine	1.23	1.30	1.91	2.00	2.00
L-Phenylalanine	2.69	2.64	3.84	4.00	3.00
L-Threonine	1.00	0.95	2.47	2.50	1.50
L-Tryptophan	0.97	1.00	0.74	1.00	1.50
L-Tyrosine	2.32	2.05	3.86	4.00	2.50
L-Valine	1.10	0.91	4.29	4.00	1.50
Glycine			4.70	5.00	5.00
L-Alanine			3.40	3.50	5.00
L-Aspartic acid			4.20	5.00	5.00
L-Cystine			0.20	0.50	2.50
L-Glutamic acid			13.60	8.00	10.00
L-Proline			6.08	5.00	5.00
L-Serine			3.17	3.00	5.00
Total	19.64	19.46	68.19	70.00	70.00
Amino acid mix				70	70
Vitamin-free casein			55	..	..
Gelatin			15	..	..
Corn oil			5	5	5
Cod liver oil			2	2	2
Dextrin, white			6	6	6
Mineral mix			4	4	4
Vitamin mix			3	3	3
alpha-Cellulose flour			8	..	..
Carboxymethylcellulose			2	10	10
Water			300	100	100

two amino acid mixtures one approximating the amino acid content of yolk-sac fry and salmon fingerlings and the other that of casein-gelatin. Each mixture was fed at the level in the control diet (table 1). The amounts of the amino acids, glycine through serine (table 1), added to the amino acid mixture exceeded the content of these respective amino acids found in the casein-gelatin control with the exception of glutamic acid and proline. These two were lowered in amounts to adjust the final amino acid mixture to 70% of the diet.

Prior to preparing the mixtures, each lot of individual amino acids was checked for purity by comparing the specific rotation values with those listed in the literature. All lots checked within 90% of recorded values with the exception of L-threonine which was of course (—) instead of (+) as listed in the handbook.

A preliminary test indicated at least 5% carboxymethylcellulose would be necessary to obtain satisfactory feeding consistency with the diet ingredients proposed and to obtain this amount it was necessary to modify the levels of some less essential components in the complete vitamin test diet (Halver, '57). An introductory 10-week feeding trial comparing the original complete vitamin-test diet with the modifications necessary to increase the inert ingredients to 11.7% of the dry weight of the diet substantiated the changes at least during this growing period (see table 2). Therefore, the corn oil and the carbohydrate components were reduced 2% each and replaced with alpha-cellulose flour. In the event that carboxymethylcellulose would inhibit growth in salmon, 2% CMC was included in the modified control diet. The mineral mixture and vitamin supplement were prepared as described previously in the vitamin studies (Halver, '57).

The amino acid test diet was prepared in a dough mixer equipped with a wire beater. To prepare 200 gm of diet, 70 gm amino acid mixture, 5 gm corn oil, 2 gm cod liver oil, 6 gm white dextrin, 4 gm mineral mixture and 3 gm alpha-cellulose flour containing the vitamin supplement were blended in the dry state until a homogeneous mixture was obtained. To these

ingredients was added 100 ml of distilled water that had been heated to 80 to 90°C. The mass was then stirred until homogeneous and until the soluble components were in solution. With the mixer in motion, 10 gm carboxymethylcellulose was added slowly and as the diet began to solidify, the speed of the blender was increased to incorporate air into the mixture. The final diet (200 gm) was about the consistency of bread dough. When stored in low form fruit jars in the refrigerator, the firmness increased and the diet could be fed easily through a garlic press without wastage. Only as much diet as needed for one week of feeding was prepared and stored at 4 to 6°C. until used.

For comparison during the feeding trials, the modified vitamin test diet was prepared by methods previously described and stored under refrigeration until used (Halver, '57).

A random sample of yolk-sac chinook salmon fry was obtained from the U. S. Fish and Wildlife Service Spring Creek Salmon Hatchery and acclimated to the experimental constant temperature (46°F.) spring water supply. After the yolk-sacs had been absorbed, the fry were taught to feed on the control diet. When all fish were feeding actively on the control diet, 6 representative random samples of 200 fish each were hand counted into 4-foot screened plastic-coated hatchery troughs supplied with apparently pathogen-free water at three gallons per minute. The fish were weighed to confirm representative weights (100 ± 2 gm), and after one day of rest and acclimation, were started on the feeding regime.

Fish were fed three times daily, 6 days weekly with no diet fed on Sundays. Bi-weekly weights were taken by one individual (GDG) throughout the feeding period by removing the entire population in a net, draining the water over a 10-second period, pouring into a tared container on a solution balance, weighing to within one gram and immediately returning to the trough. Dead fish were removed as soon as observed and the troughs were cleaned daily to prevent both the accumulation of feces and the growth of slime and fungus. Only as

much food as the fish would consume within one minute after presentation was expelled through a garlic press into the upper one-third of the trough by one individual throughout the feeding period. Equipment common to all lots was rinsed in disinfectant after each use to prevent transfer of nutrients or disease organisms from one lot to the next trough. At the 6-, 10- and 14-week weighing periods, 5 fish samples were removed, examined for possible external or internal abnormalities, and were preserved for future histological analysis.

After the 10th week of feeding, the growth curves of the two lots of fish fed the amino acid mixture corresponding to the approximate amino acid content of yolk-sac fry protein were divergent from the other groups and were discontinued in the interest of economy.

The remaining 4 troughs were continued until the 14-week feeding period was terminated. At this time, prior plans necessitated use of the spring water system to initiate the qualitative experiments to be reported in a later paper,[2] and the 4 troughs of fish were transferred to the river water system ($46 \pm 2°$F.). Unfortunately, the river system was contaminated with *Hexamitus salmonis*[3] and during the 15th week of feeding, all 4 groups exhibited a severe octomitis disease infection. At the termination of the 16-week feeding period (two weeks in river water), all fish examined showed *Hexamitus* in the gastro-intestinal tract, the fish exhibited little appetite and greater mortality was observed in the final two weeks of feeding than in the preceding 14 weeks in the spring water environment.

RESULTS AND DISCUSSION

After 14-weeks of feeding the amino acid test diet corresponding to the approximate amino acid content of the casein-gelatin control diet, no abnormal indications in appetite, growth, mortality, or post-mortem examinations of external and internal organs were observed. The growth of these fish

[2] To be published later.

[3] Synonym — *Octomitis salmonis* commonly used by fish cultural personnel.

compared favorably with those fed the modified vitamin test control diet as can be seen by examining figure 1. Mortality was normal (4%) for the 14-week feeding period, comparing favorably with that found previously in semi-purified salmon diet studies (Halver, '57). The fish in lots YSF, although growing more slowly, did not show any other gross symptom

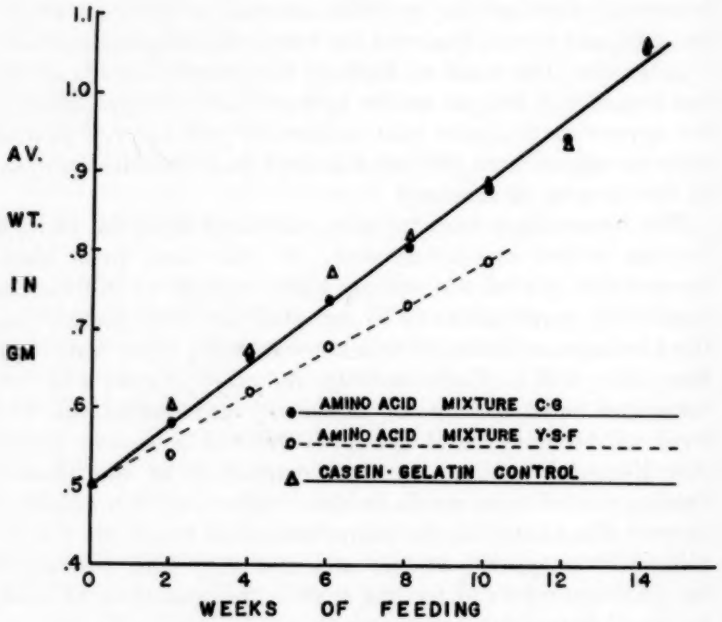

Fig. 1 Growth of salmon fingerlings on amino acid test diets.

or abnormality. At the end of this experiment (10 weeks), no pathological condition was revealed upon cursory examinations. Similar examinations indicated normal healthy fish in the lots fed either the amino acid test diet or the control diet (14 weeks).

Apparently it was not necessary to increase the caloric intake above that found in the control diets for proper utili-

zation of the free amino acids used in these mixtures (Rose, Coon and Lambert, '54; Fisher and Johnson, '56), and since the caloric intake was nearly identical for the two amino acid test diets, the discrepancy in growth with the YSF mixture must have been due to improper amino acid balance. The magnesium content of the control diet seemed adequate for utilization of free amino acids (Benton et al., '55; Heincke, Harper and Elvehjem, '55), and it was not necessary to increase the intake in order to obtain growth with the C-G amino acid mixture comparable to that of the control lots.

Feed efficiency and utilization was difficult to measure because the diet sank slowly in the trough and undoubtedly certain soluble portions leached into the water. Attempts to minimize this effect and to feed slowly enough to insure that all the diet would be consumed before it reached the bottom of the trough were undertaken but even when the greatest care was used, a small portion of the ration would be left on the bottom before the majority of the actively-feeding salmon would indicate any food refusal. Undoubtedly, a more efficient binder for the dry ingredients would still allow them to become available for digestion and assimilation, would improve the efficiency of the diet, and would furnish a more accurate measurement of food conversion and protein efficiency ratio (table 2).

Deleting the crystalline amino acids individually from the amino acid test diet C-G offers the possibility of developing specific amino acid deficiency syndromes and determining the amino acids that are indispensable for chinook salmon. This diet should furnish a lucrative tool for protein requirement studies and contribute to the determination of the quantitative amino acid requirements of salmon. The experimenter would not be shackled with the use of protein mixtures to change the dietary intake of one or more amino acids, but would have positive experimental control over each amino acid in the protein component of the diet.

TABLE 2

Growth, mortality and food conversion of chinook salmon on various amino acid test diets and on control diets

RATION	NUMBER OF FISH	INITIAL WEIGHT	FINAL WEIGHT	WEEKS OF FEEDING	PER CENT GAIN	PER CENT MORTALITY	DRY FOOD FED	CONVERSION RATIO
		gm	*gm*				*gm*	
CVTD [1]	500	0.50	0.91	10	92	2.0	365	1.8
	500	0.50	0.92	10	94	2.5	365	1.8
Control	500	0.50	0.90	10	90	2.4	360	1.8
	500	0.50	0.93	10	96	1.8	360	1.8
Control	200	0.50	1.07	14	114	4.5	200	1.9
	200	0.50	1.09	14	118	4.0	200	1.8
YSF [2]	200	0.50	0.80	10	60	5.5	160	2.8
	200	0.50	0.78	10	56	2.5	160	3.0
C-G [3]	200	0.50	1.06	14	112	4.5	265	2.5
	200	0.50	1.06	14	112	3.5	265	2.5

[1] Complete vitamin test diet (Halver, '57).
[2] Yolk-sac fry — salmon fingerling amino acid mixture test diet.
[3] Casein-gelatin amino acid mixture test diet.

SUMMARY

An amino acid test diet was formulated which would maintain chinook salmon fingerlings for at least a 14-week growing period under the experimental conditions used.

The amino acid mixture comprising the nitrogen source consisted of crystalline L-amino acids bound together with the other diet components by carboxymethylcellulose.

An amino acid mixture approximating the content of amino acids in yolk-sac fry and fingerling salmon protein failed to yield acceptable growth when compared with the above amino acid mixture or the control diet.

The use of an amino acid test diet for salmon in which the experimenter has control over each amino acid in the protein component, should simplify the determinations of the qualitative and quantitative amino acid requirements of chinook salmon.

ACKNOWLEDGMENTS

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STUDIES ON THE EFFECT OF ANTIBIOTICS ON THE INTESTINAL WEIGHTS OF CHICKS[1]

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Aside from body weight changes the most consistent effect of antibiotics on the bodies of chicks has been a reduction in the weight of the intestinal tract. Thus, Gordon ('52) observed a reduction in the weight of the small intestine of conventionally raised chicks fed antibiotics, while none was observed in animals raised in an apparently bacteria-free environment. These findings have been confirmed by Coates ('53), Coates et al. ('55), Pepper et al. ('53), and Jukes et al. ('56).

The purpose of the work presented in this report was to determine whether or not the reduction in intestinal weight preceded or followed the body weight changes, and also whether the weight of the intestines might be changed by dietary means other than antibiotic feeding.

PROCEDURE

For all the studies presented in this report chicks, either New Hampshire or White Plymouth Rock, were obtained from a commercial hatchery and placed in electrically heated battery brooders with raised wire floors at one day of age.

A corn-soybean meal diet was used in most of the studies; in one experiment a purified casein-cerelose diet was employed. These diets are given in table 1. Feed and water were supplied ad libitum.

[1] Contribution from the Department of Poultry Science, North Carolina Agricultural Experiment Station, as Journal Paper 777.

At stated intervals of time the chicks were weighed, sacrificed, and the intestines removed from the gizzard to the point of attachment of the ceca. The pancreas was removed, the intestine slit open, and the intestinal contents washed out. The intestine was then blotted on paper towels and weighed

TABLE 1

Composition of diets

INGREDIENT	CORN-SOYBEAN MEAL BASAL	CASEIN-CERELOSE BASAL
	%	%
Soybean meal (44% protein)	40.00	
Yellow corn	53.00	
Casein		25.00
Cerelose		61.00
Defluorinated rock phosphate	5.00	4.00
DL-Methionine	0.30	0.30
NaCl	0.50	0.50
Choline chloride	0.20	0.20
$MnSO_4 \cdot H_2O$	0.02	0.02
Vitamin mixture [1]	1.00	1.00
Trace mineral mixture [2]	1.00	1.00
Hydrogenated fat		3.00
Cellulose		2.00
Glycine		0.60
L-Arginine HCl		0.40
KCl		0.40
$MgSO_4 \cdot 7H_2O$		0.25

[1] Supplies per pound of feed: Vitamin A, 3000 I.U., Vitamin D, 360 I.C.U., menadione, 20 mg; alpha tocopherol acetate, 20 mg; riboflavin, 2.6 mg; thiamine·HCl, 1.6 mg; pyridoxine·HCl, 2.6 mg; Ca pantothenate, 8.4 mg; folic acid, 0.5 mg; vitamin B_{12}, 8 μg; biotin, 80 μg; niacin, 24 mg.

[2] Supplies per pound of feed: $MnSO_4 \cdot H_2O$, 100 mg; $CoCl_2 \cdot 6H_2O$, 1.0 mg; KI, 1.0 mg; $FeSO_4 \cdot 7H_2O$, 2.25 mg; $Na_2MoO_4 \cdot 2H_2O$, 1.0 mg; $CuSO_4 \cdot 5H_2O$, 2 mg.

on a triple-beam balance to the nearest 0.01 gm. The results of the intestinal weights are expressed both as absolute weights and as percentage of body weight.

The chicks used for each sample were selected on the basis of average body weights. The average body weight of the sample closely approximated the average body weight of the entire lot from which the sample was drawn.

The data were analyzed statistically using the "t" test.

RESULTS

Response of chicks to antibiotics at various ages. The first series of experiments were conducted to determine whether the intestinal response preceded or followed the body growth response. In experiment 1 the chicks were weighed at weekly intervals and samples of 20 birds were drawn for intestinal inspection at each weighing. One sample of 40 birds which had been arbitrarily divided into two lots was killed before they had been fed and the intestines removed and weighed. The results of this study are presented in table 2.

At one week of age the body weights of the birds fed antibiotics were much heavier than those not fed antibiotics. The absolute weights of the intestines were smaller and the relative weights of the intestines (percentage of body weight) were much smaller than those not fed antibiotics.

The difference between the relative weights of the small intestines at this age was significant ($P = 0.05$).

The differences between the weights of the small intestines of the treated and control birds increased at three and 4 weeks of age. At 5 weeks of age, however, there was less difference between the intestinal weights of the two groups than at 4 weeks of age.

While the first experiment clearly demonstrated that chlortetracycline reduced the weight of the small intestine very early in the life of these chicks, no conclusions can be drawn as to whether or not the intestinal weight changes preceded or followed the body weight increase, since at one week of age both phenomena had already occurred. In order to obtain more information on this question the experiment was repeated in experiment 2 and instead of weekly intervals, daily intervals were used. The results of this study are presented in table 3.

At no time in this experiment did the body weight differences between the control and treated groups approach significance. It should be noted that the chicks of the control group in experiment 2 grew much faster than those in experiment 1, reaching approximately the same weight at 5 days as those in experiment 1 reached at 7 days.

TABLE 2 [1]

The effect of chlortetracycline on the body and intestinal weights of chicks at weekly intervals

WEEKS ON EXP.	BODY WT.[2]		INTESTINAL WEIGHT		INTESTINAL WT. AS % OF BODY WT.	
	Control	Antibiotic [3]	Control	Antibiotic	Control	Antibiotic
	gm		*gm*	*gm*		
0	35 ± 0.70 [4]	35 ± 0.87	1.11 ± 0.18	1.13 ± 0.18	3.15 ± 0.34	3.19 ± 0.42
1	65 ± 2.20	82 ± 3.00	5.50 ± 0.17	5.20 ± 0.20	8.37 ± 0.52	6.35 ± 0.72
2	131 ± 8.00	164 ± 6.90	10.95 ± 0.89	10.71 ± 0.58	8.43 ± 0.49	6.57 ± 0.28
3	212 ± 17.0	268 ± 10.0	13.40 ± 1.30	11.40 ± 0.67	6.45 ± 0.43	4.36 ± 0.27
4	352 ± 16.0	412 ± 17.0	17.73 ± 0.92	15.05 ± 0.67	5.03 ± 0.10	3.48 ± 0.10
5	488 ± 21.4	546 ± 16.8	19.62 ± 0.70	17.72 ± 0.87	4.05 ± 0.12	3.24 ± 0.12

[1] Experiment 1.
[2] Twenty birds used per treatment at each time interval.
[3] Chlortetracycline, 100 mg/lb.
[4] Standard error.

TABLE 3 [1]

The effect of chlortetracycline on the body and intestinal weights of chicks at daily intervals

DAYS ON EXP.	BODY WT.[2]		INTESTINAL WT.		% INTESTINAL WT. AS % OF BODY WT.	
	Control	Antibiotic	Control	Antibiotic	Control	Antibiotic
	gm	*gm*	*gm*	*gm*		
0	40 ± 0.89 [3]	41 ± 0.99	1.67 ± 0.10	1.78 ± 0.12	4.14 ± 0.21	4.36 ± 0.31
1	42 ± 1.27	46 ± 1.03	1.83 ± 0.15	1.93 ± 0.10	4.36 ± 0.28	4.15 ± 0.19
2	47 ± 0.95	51 ± 1.45	2.66 ± 0.14	2.68 ± 0.13	5.67 ± 0.31	5.23 ± 0.22
3	53 ± 0.92	57 ± 1.63	3.19 ± 0.20	3.22 ± 0.15	6.00 ± 0.42	5.64 ± 0.26
4	51 ± 2.20	59 ± 2.00	3.95 ± 0.14	3.84 ± 0.23	7.82 ± 0.44	6.42 ± 0.20
5	62 ± 1.70	68 ± 2.70	4.72 ± 0.25	4.50 ± 0.24	7.65 ± 0.48	6.60 ± 0.21

[1] Experiment 2.
[2] Ten chicks per treatment at each time interval.
[3] Standard error.

The difference in the absolute weights of the intestines of the two groups of chicks began to be apparent on the 4th day and was much greater on the 5th day. The difference between the relative intestinal weights of the treated and control groups became apparent on the third day but this difference was not significant. On the 4th and 5th days, however, the difference was significant ($P = 0.01$).

These data, while not conclusive, strongly indicate that the effect of chlortetracycline on the intestinal tract precedes the growth effect, and suggests that the small intestine is more sensitive to antibiotics than is the total body as reflected by body weight.

Antibiotics occasionally do not produce a growth response under laboratory conditions. In the course of these studies this was demonstrated in experiment 3 (table 4). At no time in this experiment did the antibiotic stimulate growth significantly, nor did it have any effect on the weight of the small intestine. While negative results usually have little meaning, taken in connection with the previous experiments, these results could be interpreted to indicate that the lack of growth effect in this study might have been related to the lack of an effect on the small intestine.

Effect of level of antibiotic on the response of chicks. In these studies, a much higher concentration of antibiotic was used than is normally necessary to produce a growth response. It was necessary to confirm further the effect of antibiotics on the small intestine and body weight by using much lower concentrations. Accordingly experiment 4 was undertaken to study the effect of increasing concentrations of antibiotic, from 2 to 100 mg/lb., on the small intestine and body weights. In this experiment procaine penicillin was used as the antibiotic. Two types of diet were used; the corn-soybean meal used in the previous study, and the casein-cerelose diet (table 1), in order to ascertain whether or not the diet influenced the intestinal response to the antibiotic. The results of this study are presented in table 5.

TABLE 4 [1]

Lack of effect of chlortetracyline on body and small intestinal weights at various intervals

DAYS ON EXP.	BODY WT.[2]		INTESTINAL WT.		% INTESTINAL WT. AS % OF BODY WT.	
	Control	Antibiotic	Control	Antibiotic	Control	Antibiotic
	gm	*gm*	*gm*	*gm*		
4	53 ± 0.49 [3]	52 ± 1.0	3.35 ± 0.19	3.35 ± 0.15	6.35 ± 0.34	6.50 ± 0.94
12	110 ± 7.40	113 ± 3.9	7.68 ± 0.32	7.37 ± 0.38	7.16 ± 0.50	6.52 ± 0.27
17	165 ± 6.60	169 ± 11.4	9.63 ± 0.62	8.86 ± 0.55	5.80 ± 0.27	5.33 ± 0.36
22	214 ± 13.6	227 ± 13.2	10.55 ± 0.81	10.90 ± 0.50	4.92 ± 0.14	4.84 ± 0.19
27	292 ± 22.9	304 ± 15.1	12.46 ± 0.89	13.22 ± 0.67	4.01 ± 0.17	4.40 ± 0.21
33	411 ± 20.6	426 ± 26.1	15.60 ± 0.75	15.69 ± 0.84	3.81 ± 0.17	3.72 ± 0.18

[1] Experiment 3.
[2] Ten chicks per treatment at each time interval.
[3] Standard error.

TABLE 5 [1]

Effect of increasing levels of penicillin on body and intestinal weights of chicks fed two types of diet

PROCAINE PENICILLIN	BODY WT.[2]		INTESTINAL WT.		INTESTINAL WT. AS % OF BODY WT.	
	Cas.[3]	Soy.[4]	Cas.	Soy.	Cas.	Soy.
mg/lb.	*gm*	*gm*	*gm*	*gm*		
0	502 ± 14 [5]	631 ± 21	20.23 ± 1.73	19.00 ± 1.4	4.06 ± 0.30	3.05 ± 0.14
2	489 ± 27	670 ± 22	16.39 ± 1.4	15.56 ± 0.99	3.47 ± 0.22	2.76 ± 0.17
10	517 ± 20	656 ± 19	14.37 ± 1.4	15.83 ± 1.4	2.83 ± 0.19	2.41 ± 0.09
25	520 ± 19	697 ± 19	12.65 ± 0.94	17.59 ± 0.42	2.42 ± 0.14	2.52 ± 0.03
50	550 ± 25	697 ± 19	14.56 ± 0.73	18.39 ± 0.62	2.65 ± 0.10	2.65 ± 0.08
100	593 ± 14	702 ± 22	14.21 ± 0.45	15.71 ± 0.86	2.40 ± 0.07	2.26 ± 0.08

[1] Experiment 4.
[2] Twenty 6-week-old chicks per treatment.
[3] Cas. = Casein-cerelose diet.
[4] Soy. = Soybean meal-corn diet.
[5] Standard error.

On the casein diet only the highest level of penicillin fed produced a significant body weight response ($P = 0.01$). On the other hand, the intestinal weight response approached significance at the lowest level fed ($P = 0.10$), and was significant at 10 mg of penicillin per pound ($P = 0.01$) and at the higher concentrations.

On the corn-soybean meal diet, penicillin at 25 mg/lb. produced a significant body growth response ($P = 0.05$), while the 10 mg/lb. level was sufficient to produce a significant intestinal response ($P = 0.05$).

The results of this study clearly demonstrated that on either a casein-cerelose or a corn-soybean meal diet, the antibiotic fed at a level too low to produce a body growth response, produced a growth-retarding effect on the small intestine. This finding again illustrates that the small intestine is more sensitive to antibiotic feeding than is the total body as reflected by weight. However, these data cast some doubt on any causal relation between lowered intestinal weight and increased body weight.

Effect of alfalfa meal and wheat bran on the response of chicks to antibiotics. In order to determine the effect of fibrous feedstuffs and the possible relationship between the effects of fibrous materials and antibiotics on the weight of the intestinal tract, experiment 5 was conducted in which wheat bran and alfalfa were introduced into the corn-soybean meal diet at the expense of corn. The results of this study are presented in table 6.

Neither 10% dehydrated alfalfa meal nor 10% wheat bran had any significant effect on body weight or intestinal size. Penicillin significantly increased the body weights of chicks in all three groups. The absolute intestinal weights were decreased in all three groups by the addition of penicillin; however, only the decrease in the basal group was significant ($P = 0.05$). The differences in the relative weights of the small intestine caused by feeding penicillin were highly significant in all three lots. These results indicate that the inclu-

[illegible] Soy. = Soybean meal-corn diet.
[illegible] Standard error.

TABLE 6 [1]

Effect of wheat bran, alfalfa, and penicillin on body and intestinal weights of chicks

TREATMENT	BODY WT.[2]		INTESTINAL WT.		INTESTINAL WT. AS % OF BODY WT.	
	Control	Antibiotic [3]	Control	Antibiotic	Control	Antibiotic
	gm	*gm*	*gm*	*gm*		
Basal	262 ± 9.2 [4]	289 ± 9.2	13.66 ± 0.48	11.83 ± 0.54	5.28 ± 0.19	4.08 ± 0.13
Wheat bran, 10%	243 ± 7.8	294 ± 7.7	13.72 ± 0.55	12.43 ± 0.27	5.66 ± 0.13	4.26 ± 0.10
Alfalfa, 10%	250 ± 7.3	279 ± 7.6	13.39 ± 0.51	12.00 ± 0.60	5.36 ± 0.14	4.33 ± 0.18

[1] Experiment 5.
[2] Twenty 3-week-old chicks per treatment.
[3] Forty milligrams procaine penicillin/lb.
[4] Standard error.

TABLE 7 [1]

Effect of penicillin on the growth and intestinal weights of chicks fed unheated soybean meal

TREATMENT	BODY WT.[2]		INTESTINAL WT.		INTESTINAL WT. AS % OF BODY WT.	
	Control	Antibiotic [3]	Control	Antibiotic	Control	Antibiotic
	gm	*gm*	*gm*	*gm*		
Basal	253 ± 9.7 [4]	288 ± 9.8	13.62 ± 0.62	11.60 ± 0.52	5.38 ± 0.15	4.04 ± 0.14
Unheated soybean meal,10%	224 ± 8.7	299 ± 8.9	13.32 ± 0.37	13.04 ± 0.47	5.95 ± 0.23	4.38 ± 0.17

[1] Experiment 6.
[2] Twenty 3-week-old chicks per treatment.
[3] Two hundred milligrams penicillin/lb.
[4] Standard error.

sion of fibrous material does not cause an increase in intestinal weight nor does it alter the effect of penicillin on the weight of the small intestine.

Effect of unheated soybean meal on response of chicks to antibiotics. It was shown by Borchers ('57) that high levels of antibiotics overcame the growth-retarding effects of unheated soybean meal in rats. Experiment 6 was, therefore, conducted to determine the effect of high levels of penicillin on the growth retardation and intestinal weight changes in chicks fed 10% unheated soybean meal in place of 10% of the heated soybean in their ration. The results of this study are presented in table 7.

Unheated soybean meal fed at a level of 10% significantly lowered the body weight of chicks compared with those chicks receiving only heated soybean meal ($P = 0.05$). The addition of 200 mg of procaine penicillin per pound of feed significantly increased the body weights of both the chicks fed all heated and those fed 10% unheated soybean meal in their diet. The effect of the antibiotic on the body weights of the group receiving 10% unheated soybean meal in their feed was the most striking, however. The inhibiting effect of the unheated soybean meal was completely counteracted by the antibiotic.

Again the antibiotic significantly reduced the intestinal weights of the chicks fed only heated soybean meal ($P = 0.05$). Unheated soybean meal did not increase the absolute weights of the small intestines of the chicks as compared to the control groups nor did penicillin significantly lower the absolute weights of the small intestines of the chicks fed unheated soybean meal. The relative intestinal weights of both groups, however, were reduced by the antibiotic. This relative reduction was highly significant ($P = 0.01$). The substitution of 10% unheated soybean meal significantly increased the relative size of the small intestine ($P = 0.05$) while the addition of antibiotic to the unheated soybean meal diet reduced the relative intestinal weight till it was not significantly different from that in the control group fed the antibiotic.

TABLE 8 [1]

Effect of penicillin on growth and intestinal weights of chicks fed increasing levels of unheated soybean meal

UNHEATED SOYBEAN MEAL	BODY WT.[2]		INTESTINAL WT.		INTESTINAL WT. AS % OF BODY WT.	
	Control	Antibiotic [3]	Control	Antibiotic	Control	Antibiotic
%	*gm*	*gm*	*gm*	*gm*		
0	367 ± 19 [4]	404 ± 18	13.1 ± 0.98	13.4 ± 1.15	3.56 ± 0.20	3.01 ± 0.17
10	337 ± 21	404 ± 28	14.6 ± 1.1	12.6 ± 0.99	4.39 ± 0.28	3.14 ± 0.17
30	250 ± 25	326 ± 19	10.2 ± 0.56	12.8 ± 0.89	4.16 ± 0.26	3.71 ± 0.14
40	282 ± 25	288 ± 21	12.1 ± 1.4	10.4 ± 0.73	4.20 ± 0.20	3.72 ± 0.17

[1] Experiment 7.
[2] Ten 4-week-old chicks per treatment.
[3] Two hundred milligrams procaine penicillin/lb.
[4] Standard error.

These results indicate that a high level of penicillin will counteract the growth-retarding effect of 10% unheated soybean meal and thus confirm the findings of Borchers and extend these findings to chicks. In this experiment substitution of 10% unheated soybean meal in the diet increased the relative weights of the small intestine but not the absolute weights. Since the small intestine does not grow at the same rate as the whole body these results are difficult to interpret in terms of actual effect on the small intestine.

These lots were repeated, therefore, in experiment 7. In addition to the substitution of 10% unheated soybean meal in the diet, 30 and 40% were also substituted in place of heated soybean meal in order to determine the effectiveness of high levels of antibiotic in overcoming the inhibition of greater amounts of unheated soybean meal. The results of this study are presented in table 8.

While the substitution of 10% unheated soybean meal in the diet again resulted in decreased growth, this decrease was not significant. It should be noted, however, that the addition of high levels of penicillin again increased the body weight of the chicks to equal that of the control lot fed penicillin. The feeding of 10% unheated soybean meal also caused an increase in the intestinal weight. The increase in the absolute intestinal weight of the intestine was not significant, but that of the relative weight was ($P = 0.05$). The addition of penicillin to the lot fed 10% unheated soybeans reduced both the absolute and relative weights of the small intestine to approximately that of the control lots fed penicillin. The substitution of 30 and 40% unheated soybean meal in the ration further reduced the growth of the chicks. The antibiotic increased the growth of the chicks fed 30% unheated meal but not those fed 40% unheated meal. While the higher levels of unheated soybean meal reduced the absolute weights of the small intestine in comparison with the 10% level, they did not significantly change the relative weights. Furthermore, while the antibiotic significantly increased the growth of the chicks

fed 30% unheated soybean meal, it did not reduce the absolute weight nor the relative weights of the small intestine significantly. This finding again casts some doubt on any causal relationship between the intestinal weight reduction and body weight increases brought about by feeding antibiotics.

SUMMARY

The results of the experiments presented in this report indicate that the small intestine is more sensitive to the antibiotics used than is the whole body as reflected by body weight because (1) the small intestine decreased in weight before an increase was noted in body weight, and (2) lower levels of the antibiotic which did not increase body weight caused a decrease in intestinal weight.

High levels of penicillin completely counteracted the body growth retardation and relative intestinal weight stimulation of unheated soybean meal fed at a level of 10% of the total diet, but did not completely counteract these effects when the level of unheated soybean meal was increased to 30 or 40%.

The inclusion of fibrous materials, alfalfa and wheat bran, at a level of 10% of the total diet did not significantly affect the response of the small intestine or body weight to penicillin.

It is suggested that the findings presented in this report cast some doubt on the possibility that there exists a causal relationship between the effect of antibiotics on the small intestine and the growth response usually observed when antibiotics are fed.

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THE EFFECT OF RAW SOYBEAN MEAL AND TRYPSIN INHIBITOR DIETS ON PANCREATIC ENZYME SECRETION IN THE RAT [1]

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It has been amply demonstrated that heat treatment of soybean protein results in a protein of greatly improved nutritional value (Osborne and Mendel, '17; Hayward, Steenbock and Bohstedt, '36; Evans, McGinnis and St. John, '47). Although a number of mechanisms have been proposed to explain the improvement by heat, the results have been conflicting.

The observation that raw soybean contained a heat labile trypsin inhibitor (Ham and Sandstedt, '44; Bowman, '44; Kunitz, '45, '46) led to the suggestion that growth depression was the result of incomplete intestinal proteolysis, which limited the availability of certain amino acids, especially methionine (Ham et al., '45; Borchers, Ackerson and Mussehl, '48a; Almquist and Merritt, '51a; '51b). However, Westfall, Bosshardt and Barnes ('48), with concentrates of the soybean trypsin inhibitor and Klose et al. ('48), with inhibitor preparations from lima beans were able to produce growth inhibition equivalent to that with the whole meal when fed with adequately supplemented hydrolyzed casein. Hill et al. ('53) were unable to prevent growth depression in chicks fed raw soybean when the diet was fortified with adequate levels of essential amino acids. Consequently, it was felt that the

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trypsin inhibitor acted to inhibit growth other than through the effect on intestinal proteolysis.

Recently a toxic protein, soyin (Liener and Pallansch, '52; Liener, '53), has been obtained from raw soybean fractions associated with the antitrypsin which apparently acts in a non-specific manner to limit food intake in the rat and, as a result, promotes growth depression. Certain evidence has accumulated which indicates that the antitrypsin or some other factor in raw soybeans exerts a physiological effect involving the pancreas.

Chernick, Lepkovsky and Chaikoff ('48) reported that chicks fed raw soybean meal developed hypertrophic pancreases which contained abnormally high concentrations of trypsinogen. It was suggested that these changes were the result of a reaction to the soybean antitrypsin.

Further investigation [3] showed that rats fed raw soybean meal diets excreted feces containing excessive amounts of proteolytic activity. Consumption of heated soybean or casein diets failed to increase the proteolytic enzymes. Since an increase in intestinal proteolytic activity would not be expected in the presence of the antitrypsin, it became the purpose of this investigation to study the effect of whole unheated soybean as well as soybean trypsin inhibitor on the pancreatic enzymes secreted into the small intestine of the rat.

EXPERIMENTAL

Mature female rats of the Long-Evans strain were used throughout the experiments and were housed individually in raised bottom screen cages.

A procedure of trained feeding was employed which adapted the rat to eat its daily food requirement within two hours each morning. This routine provided animals which had eaten similar amounts of food at comparable periods of digestion, as well as allowing a means for observing the degree of discharge of pancreatic enzymes at the time intervals

[3] Lepkovsky, S., unpublished data.

studied. Generally, two weeks of training were required before the animal weight stabilized with another week or so necessary to regain the lost weight. Barker ('49) could show no difference in growth or oxygen consumption in rats fed during the day when compared to rats fed for a limited period at night, so apparently, the adjustment to the altered feeding habits was complete.

From one week to 10 days preceding an experiment, rats which had been trained on stock diet were fed the basic heated soybean diet. The composition of the diet is shown in table 1. Modifications of the basic diets were generally at the expense of the soybean meal.

TABLE 1

Composition of basic diets

COMPONENT	RAW SOY	HEATED SOY
	%	%
Cerelose	40	40
Raw soybean meal[1]	50	..
Heated soybean meal[2]	..	50
Soybean oil	4	4
Sardilene oil[3]	1	1
Vitamin mix[4]	1	1
Salt mixture[5]	4	4
	100	100

[1] Ottawa Mandarin soybeans purchased from Northrup King and Co., Minneapolis, Minnesota. The beans analyzed about 43% protein, 5% moisture and 20% crude fat.

[2] The whole soybean was autoclaved for 10 minutes at 20 pounds pressure, then dried overnight at 70°C. and ground in a mill.

[3] Sardilene provided 2250 U.S.P. units vitamin A and 300 International chick units vitamin D per gram.

[4] The vitamin mix was made up with cerelose and, at 1% of the diet, provided in milligrams per kilogram: thiamine, 2; riboflavin, 3; calcium pantothenate, 20; inositol, 100; biotin, 0.1; folic acid, 0.2; niacinamide, 10; vitamin B_{12} (as a 0.1% triturate), pyridoxine, 2.5. Choline was added to the vitamin mix at the time a diet was prepared so as to provide 500 mg per kilo.

[5] Salts were commercially obtained from Nutritional Biochemicals, Cleveland, Ohio, and were compounded after Hubbell, R. B., L. B. Mendel and A. J. Wakeman, J. Nutrition, *14:* 273, 1937.

On the day of an experiment, the rats were weighed and divided into two groups; one group was fed the usual heated soybean diet and the other group the raw soybean diet for the two-hour period. Spilled food was collected on papers and an accurate record of food consumption for that day was kept. Pairs of rats were then killed at intervals immediately after eating (zero hour), 3 hours, 6 hours, and 9 hours post-feeding. Animals fed their respective diets on the previous day and killed prior to being fed the day of the experiment were designated as fasted animals.

At autopsy, stomach contents and the intestinal contents from the upper end of the duodenum to the entrance of the cecum were removed completely from the slit intestine. Pancreas tissue was dissected grossly and cleaned of mesentery and fat as much as possible with the aid of a binocular. Samples were frozen in dry ice, lyophilized and weighed for dry weight values before analytical determinations were made. Weighed samples of the powdered material were homogenized in water and amylase, lipase, and trypsin analyses were performed. The concentration of the material depended upon the enzyme determined, but was generally from 0.1 to 5 mg for the intestinal contents and 0.05 to 5 mg for the pancreas.

Amylase activity was determined by a slight modification of the photometric method of Smith and Roe ('49). A 15-minute incubation at 37°C. was used rather than 30 minutes. The change necessitated multiplying results obtained by two so that they would correspond to the authors' definition of an amylase unit. An amylase unit was defined as "the amount of enzyme, that under the conditions of the procedure, with 60 mg of starch present will hydrolyze 10 mg of starch in 30 minutes to a stage where no color is given with iodine at 620 mu."

Lipase concentration was measured by a modification of the method described by Minard ('53). A suitable buffer[4] was made up in 0.08 M calcium acetate and adjusted to pH 8.5.

[4] Sigma 7–9.

Prior to using, enough bile salts [5] were added to the buffer to give a final concentration in the digestion flask of 0.2% salts. To 10 ml of the buffer were added the enzyme and 3 ml of substrate composed of an emulsion of 15% olive oil, 1% sodium cholate and 5% dextrose. Following a 60-minute incubation at 37°C. (with agitation), activity was stopped with 5 ml of a 9:1 alcohol-ether mixture, and the contents washed into a 400 ml beaker containing about 200 ml of water. The liberated fatty acids were titrated with 0.1 N sodium hydroxide using a glass electrode and an end point of pH 9.3. A similarly prepared flask containing the inactivated enzyme served as a blank. Results were expressed in milliequivalents of acid liberated per hour of incubation.

The method used for proteolytic enzyme activity was a modification of Anson's ('38) and measured trypsin and chymotrypsin as well as small amounts of amino and carboxypeptidases. However, for simplicity, the term "trypsin activity" has been used to denote the total proteolytic activities. A hemoglobin substrate at pH 7.6 was prepared according to Orringer, Lauber and Hollander ('50). Only 1 ml of the trichloroacetic acid filtrate plus 4 ml of water was used for color development. This kept the blank reading low, yet allowed a good color development from the digested sample. The enzyme activity was expressed as milliequivalents of tyrosine released per 10 minutes incubation at 37°C. Pancreas trypsinogen was activated by a 45-minute incubation at 37°C. with 1 ml of 0.5% solution of commercial duodenum powder [6] prior to addition of the substrate.

Pepsin activity was measured in the same way as trypsin with the exception that the substrate (Orringer et al., '50) was not denatured by urea and prior to use was adjusted to pH 1.7 with hydrochloric acid. Pepsin was expressed as milliequivalents of tyrosine released per 10 minutes incubation.

[5] Armour.

[6] Viodenum, obtained from the Viobin Corporation, Monticello, Illinois.

The trypsin inhibitor concentrate preparation was modified somewhat from that described by Borchers et al. ('48b). One hundred grams of ground, unextracted raw soybeans were added to 10 volumes of water and the pH adjusted to 4.2 with hydrochloric acid. After leaving overnight, the clear supernatant was removed by centrifugation. To the supernatant were added 30 gm of ammonium sulfate per 100 ml and the precipitate was collected. The ammonium sulfate precipitation was repeated twice. The precipitate was dissolved in a minimum of water and dialyzed against tap water for 6 hours at room temperature. Final precipitation was with cold acetone added to form a 70% acetone solution. After washing with ether, the material was dried in a vacuum desiccator.

RESULTS AND DISCUSSION

The effect of raw and heated soybean meal on pancreatic enzyme secretion

The influence of unheated and heated soybean diets on amylase, lipase, and trypsin activity in the pancreas and small intestine is shown in figure 1. Since the average amount of intestinal contents from the two groups was similar, enzyme activities were expressed as units of total activity in the small intestine at the various periods. Pancreas data were presented as units of enzyme activity in the dry pancreas per 100 gm body weight.

Shortly after eating the raw soybean diet there occurred an increase in the intestinal level of amylase and lipase which reached a maximum of three or 4 times that produced by the heated meal diet. The sharp decline in activity at 9 hours post-feeding appeared to be due to insufficient material passing into the small intestine to continue the stimulation. For some unexplained reason, the group of animals in the 9-hour period consumed only about one-half the food eaten by the other animals receiving raw soybean. As a result, no solids were present in the stomachs and only small amounts were found in the intestine at time of autopsy. The excessive enzyme

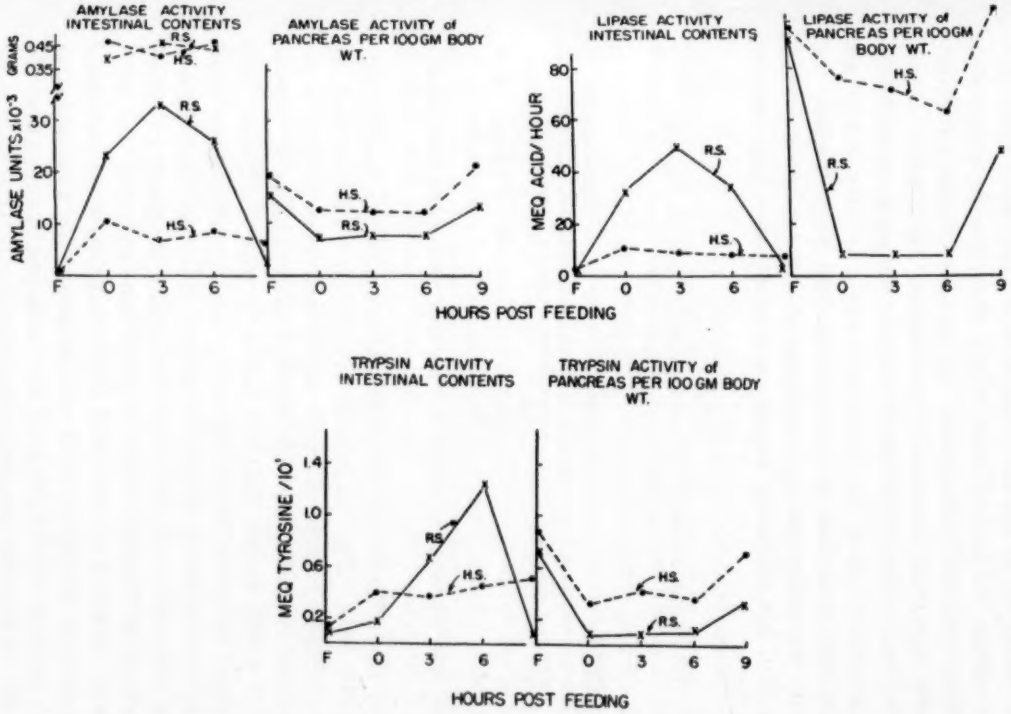

Fig. 1 The effect of raw and heated soybean meal diets on the intestinal and pancreatic amylase, lipase and trypsin activities. R.S. = basic raw soybean diet; H.S. = basic heated soybean diet; x – – – x = weight of raw soybean intestinal contents; o – – – o = weight of heated soybean intestinal contents. Each point at zero, 3, and 6 hours represents the average of the results from 6 animals. The 9 hour and fasting intervals are analyses from two rats each.

concentration in the intestine was mirrored by a consistently greater depletion of active enzymes remaining in the pancreas when compared to the activities in the pancreases from animals eating the heated meal. This depletion of pancreatic enzymes remained throughout the periods of high intestinal activity and was especially pronounced for lipase, whose activity was reduced to about 10% of the fasting concentration.

A consideration of the intestinal trypsin activity of animals on the raw meal showed that immediately after feeding, the activity was somewhat less than that of rats eating the heated bean diet, but increased rapidly and by 6 hours had risen to

TABLE 2

Total pepsin activity of the stomach contents from rats fed raw and heated soybean meal diets[1]

PERIOD POST-FEEDING	RAW SOYBEAN	HEATED SOYBEAN
hours	*milliequivalents tyrosine/10 min. incubation*	
0	0.185	0.165
3	0.195	0.180
6	0.225	0.185

[1] Each figure represents an average of 4 individual analyses for each period.

nearly three times the maximum activity of the heated soybean-fed rats. The pancreatic trypsinogen (determined as active trypsin) dropped upon eating and remained at a reduced concentration throughout the 6 hours observed. Therefore, it would appear that the initial trypsin discharged into the small intestine following eating was insufficient to completely neutralize the trypsin inhibitor. However, the response of the pancreas to the raw soybean rapidly produced an excess of intestinal trypsin which negated any effect of the antitrypsin. Samples of intestinal contents from three zero-hour periods were incubated with enterokinase [7] for 45 minutes at 37°C. prior to the addition of the hemoglobin substrate. No increase in activity occurred. So a deficiency of enterokinase

[7] See footnote 6, page 273.

was not considered to be involved in the initially low trypsin activity of the intestinal contents.

The results of pepsin analyses of the stomach contents from both groups of rats are shown in table 2. The factor in raw soybean seemed to be specific for stimulating the pancreas, since no appreciable difference in pepsin activity existed between the two groups when measured over the 6 hours.

The effect of soybean trypsin inhibitor concentrate and crystalline soybean trypsin inhibitor on pancreatic enzyme secretion

In an attempt to determine whether the soybean trypsin inhibitor was responsible for the pancreatic stimulation, small amounts of crude trypsin inhibitor concentrate were added to the basic heated soybean diet. The concentrate had about 25 times more antitrypsin activity than the original whole meal (as measured by the method of Borchers, Ackerson and Sandstedt, '47). When added to the basic heated soybean diet at 1, 2 and 4%, the material provided trypsin-inhibitor activity of about one-half, one and two times the inhibitor activity of the raw soybean diet. The rats were handled as previously described with the exception that only the zero-, 3-, and 6-hour intervals were studied. Figure 2 shows that the three levels of crude inhibitor were nearly as effective as raw soybean in increasing enzyme activity in the intestinal contents. The pancreases consistently reflected the increased intestinal activity by showing an enzyme content as low as that produced by the raw meal. At 1%, the inhibitor preparation appeared not to promote a full response from the pancreas. This was apparent from the depressed activity of intestinal lipase and only a slight exaggeration in the intestinal trypsin activity. Amylase, however, appeared to be increased about the same at all levels of the concentrate. A more conclusive indication of incomplete stimulation by 1% of the inhibitor may be seen from the residual pancreatic enzyme concentration. The activities remained at a level higher than that of the fully

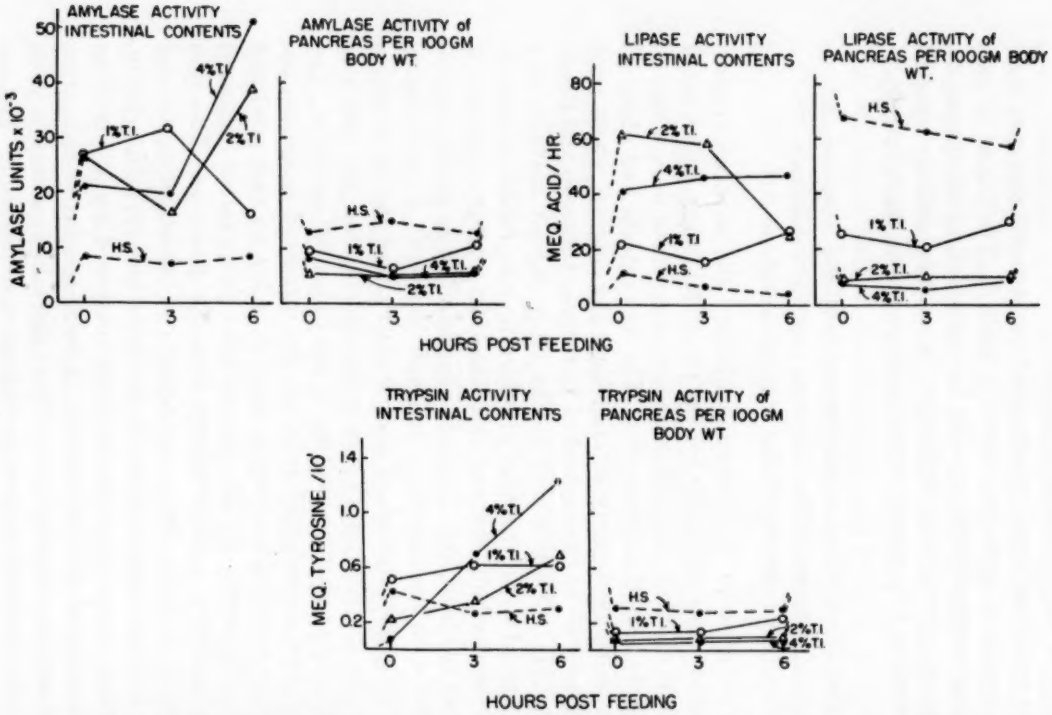
AMYLASE ACTIVITY INTESTINAL CONTENTS
AMYLASE ACTIVITY of PANCREAS PER 100GM BODY WT
LIPASE ACTIVITY INTESTINAL CONTENTS
LIPASE ACTIVITY of PANCREAS PER 100GM BODY WT.
AMYLASE UNITS x 10⁻³
MEQ. ACID / HR.
4% T.I.
2% T.I.
1% T.I.
H.S.
HOURS POST FEEDING
TRYPSIN ACTIVITY INTESTINAL CONTENTS
TRYPSIN ACTIVITY of PANCREAS PER 100GM BODY WT
MEQ. TYROSINE /10'
HOURS POST FEEDING

Fig. 2 The effect of different concentrations of crude soybean trypsin inhibitor on the intestinal and pancreatic amylase, lipase, and trypsin activities. T.I. = crude trypsin inhibitor; H.S. = basic heated soybean diet. Each point represents the analyses from one animal at each interval for the inhibitor concentrates and two animals for the heated soybean group.

stimulated animals, yet lower than the concentration remaining after ingestion of the heated meal diet. Therefore, the response evoked by the inhibitor concentrate must not be "all or none," but may be of a graded nature up to some optimal concentration of the stimulating agent.

Liener ('51, '53) had shown that crude trypsin-inhibitor preparations contained at least one contaminating substance, soyin, which may also act as a growth inhibitor. Since the diet fed contained the crude preparation, there was no way of knowing whether the enzyme response was due to the trypsin inhibitor or to associated impurities. Consequently, a heated soybean diet was prepared containing 0.5% crystalline soybean trypsin inhibitor.[8] At this level of inhibitor, the antitrypsin activity of the diet was about 1.5 times more than a similarly compounded diet containing 2% of the crude inhibitor preparation. Since the 2% crude trypsin-inhibitor diet had been shown to be capable of producing pancreatic secretory response, any stimulation by the crystalline inhibitor should distinguish between an effect due to an impurity and one due to the trypsin inhibitor *per se*.

Figure 3 presents the results obtained. It is apparent that the crystalline soybean trypsin inhibitor was as effective in exciting a pancreatic discharge as the 2% crude inhibitor. Both intestinal amylase and lipase activity were increased and the pancreas showed the characteristic extensive depletion of residual enzymes. Although the pancreatic trypsin depletion produced by the crystalline inhibitor was equivalent to that seen with the 2% inhibitor diet, the intestinal trypsin activity appeared very much like that produced by the heated soybean. Inasmuch as the crystalline antitrypsin provided at least 1.5 times the trypsin inhibiting activity of the 2% diet, it was surprising that no depression in the active trypsin of the intestinal contents at zero hour was obtained.

The crystalline inhibitor was isolated according to the method of Kunitz ('46) [9] and recrystallized 5 times with a

[8] Purchased from Worthington Biochemical Corporation, Freehold, New Jersey.
[9] Manufacturer's specifications.

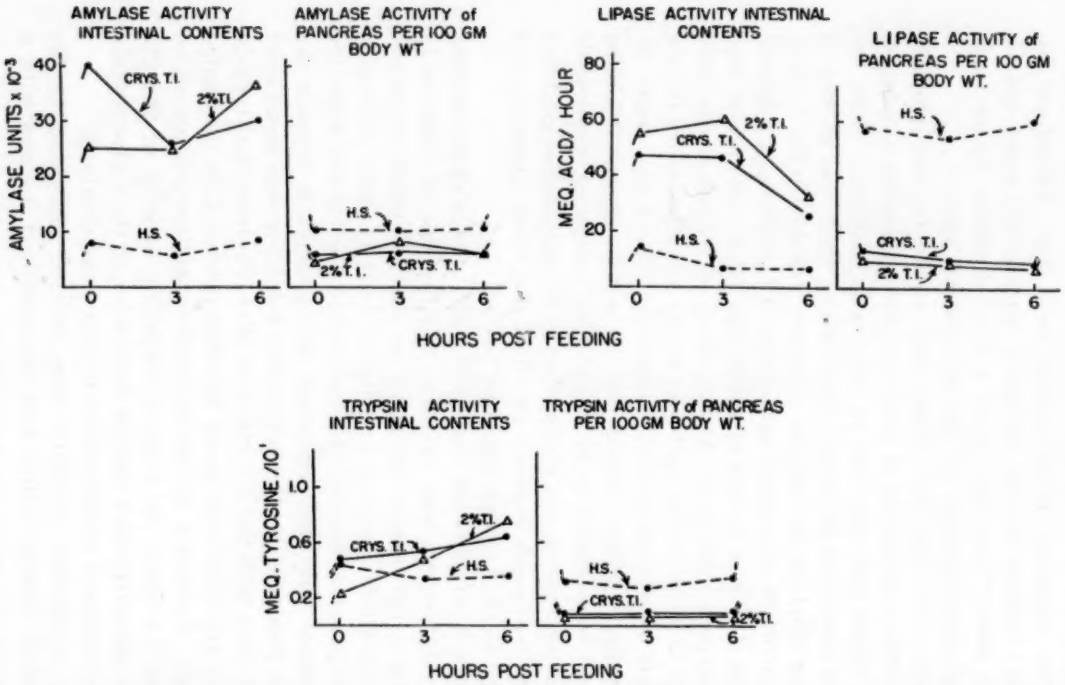
AMYLASE ACTIVITY INTESTINAL CONTENTS
AMYLASE ACTIVITY of PANCREAS PER 100 GM BODY WT
LIPASE ACTIVITY INTESTINAL CONTENTS
LIPASE ACTIVITY of PANCREAS PER 100 GM BODY WT.
AMYLASE UNITS x 10⁻³
MEQ. ACID/ HOUR
CRYS. T.I.
2% T.I.
H.S.
HOURS POST FEEDING
TRYPSIN ACTIVITY INTESTINAL CONTENTS
TRYPSIN ACTIVITY of PANCREAS PER 100 GM BODY WT.
MEQ. TYROSINE /10'
HOURS POST FEEDING

Fig. 3 The effect of crystalline soybean trypsin inhibitor on the intestinal and pancreatic amylase, lipase, and trypsin activities. Cryst. T.I. = 0.5% crystalline soybean trypsin inhibitor; 2% T.I. = 2% crude trypsin inhibitor concentrate; H.S. = basic heated soybean diet. Each point represents the average of analyses from two rats per interval.

final crystallization from ethyl alcohol. The material thus used must have been as pure as the crystalline inhibitor employed by Liener ('51) which failed to give a toxic reaction when injected into rats and was considered to be free of the toxic component, soyin. The evidence, therefore, indicates that the factor in raw soybean stimulating pancreatic enzyme secretion is the soybean trypsin inhibitor.

The data establish that the soybean trypsin inhibitor evokes an augmented discharge of enzymes from the rat pancreas. At the present time, there is little reason to believe that the increase in intestinal enzyme activity might be due to a retention and accumulation of enzyme in the small intestine, rather than to an actual increased rate of secretion. Therefore, the rise in intestinal enzyme concentration which was accompanied by a continuously depleted state of the enzymes in the pancreas suggests that the enzymes were discharged at a rate equal to their synthesis with none accumulating. If this is the case, the rat pancreas appears able to maintain a substantially increased rate of synthesis for at least 6 hours and possibly longer. Lin and Grossman ('52) had observed that pancreatic fistulated dogs were able to respond to prolonged injections of pancreozymin for periods up to 14 hours with no obvious exhaustion of pancreatic enzymes. Extended over periods of days or weeks, a secretion of this nature might result in a substantial removal of endogenous nitrogen.

The response by the pancreas to the soybean antitrypsin results, after only a short deficient period, in excessive quantities of intestinal proteolytic activity. Consequently, it would seem unlikely that, in the rat, growth inhibition could result from insufficient intestinal proteolysis. The short period of time when protein hydrolysis might not be optimum would quickly be counteracted by the interval when excessive trypsin activity is present. The increased proteolytic activity apparently does not result from an exaggerated response incurred by the trained feeding regime. It had been noticed for some-

time [10] that rats fed raw soybean excreted feces containing 4 or 5 times the normal amount of proteolytic activity. The increased activity would remain for several weeks if the animals continued on raw soybean diets. It is possible that the influence of the antitrypsin on the pancreas may be more important nutritionally than any effect it might exert upon intestinal proteolysis.

SUMMARY

The influence of raw soybean and crystalline trypsin inhibitor on pancreatic enzyme secretion in the small intestine of the rat was investigated.

Animals that had been trained to eat their daily food requirement in two hours were used to follow the enzyme response at different intervals after eating. The results showed that immediately after ingesting a 50% raw soybean meal diet intestinal amylase and lipase activity increased. By three hours, the concentration of enzyme was three or 4 times that produced with the heated meal. The enhanced intestinal enzyme activity was reflected by a concomitant depletion of pancreatic enzymes. Intestinal trypsin activity was low immediately after eating but increased steadily and by 6 hours was three times the normal concentration. The low initial activity apparently resulted from inactivation by the inhibitor, while the later rise in activity was due to pancreatic secretory stimulation. A crude preparation of soybean antitrypsin as well as crystalline soybean inhibitor also produced the pancreatic response, so the stimulating agent in raw soybean must be the antitrypsin. Pepsin secretion was unaffected by raw soybean which suggested a specific effect of the inhibitor on the pancreas.

The high level of intestinal trypsin produced would seem to argue against the concept that intestinal proteolysis in the rat is seriously impaired by the soybean trypsin inhibitor.

[10] See footnote 3, page 270.

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THE EFFECT OF RAW SOYBEAN MEAL AND TRYPSIN INHIBITOR DIETS ON THE INTESTINAL AND PANCREATIC NITROGEN IN THE RAT

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It has been proposed that the soybean trypsin inhibitor is responsible for the poor biological value of unheated soybean protein. Borchers, Ackerson and Mussehl ('48) showed that good nutritional value from soybean protein was dependent upon heat destruction of the antitrypsin. Melnick, Oser and Weiss ('46) demonstrated *in vitro* that in the presence of trypsin inhibitor methionine was released from intact protein too late to be utilized with the rest of the amino acids. This appeared to explain why methionine was so effective in counteracting the growth depression produced by the unheated bean. However, Liener and Fevold ('49) and Riesen et al. ('47) were unable to confirm this observation and failed to show a selective delayed release of methionine, although the liberation of all the amino acids was retarded by the antitrypsin. Almquist and Merritt ('51a, '51b, '53) provided evidence with the chick that the action of the soybean inhibitor was a general one and not necessarily specific for inducing a deficiency of methionine. Thus when the inhibitor was present any amino acid marginal in the diet became limiting for adequate growth. Consequently, it was felt that the etiology of the growth inhibition in the chick was through an impairment of protein hydrolysis brought about by the soybean antitrypsin. The growth depression noted, however, when trypsin

inhibitor concentrates were fed with hydrolyzed proteins could not be explained on the basis of the inhibitor's effect on normal intestinal proteolysis.

Liener ('53) suggested that the opposing views might be reconciled if one considered that part of the growth depression was due to intestinal trypsin inhibition and part the result of the toxic protein, soyin, which had been shown to interfere with normal appetite. Therefore, when crude concentrates of antitrypsin were fed with hydrolyzed protein, growth depression was not induced by the inhibitor, but by soyin which was thought to contaminate the impure antitrypsin preparation. On the other hand, information obtained in this laboratory (Lyman and Lepkovsky, '57) failed to demonstrate any prolonged inhibition of intestinal trypsin in rats fed raw soybean or trypsin inhibitor. Moreover, instead of a deficiency of intestinal enzymes, quantities in excess of normal were secreted in response to the soybean trypsin inhibitor.

Therefore, it became the purpose of this investigation to study the nitrogen in the pancreas and small intestine of rats fed raw and heated soybean diets in order to see whether the enzyme secretion might represent a significant nitrogen loss to the rat.

EXPERIMENTAL

The handling of the rats and samples, i.e., diets, trained feeding procedures, removal and analyses of intestinal contents and pancreas and general experimental procedures were described in a preceding paper (Lyman and Lepkovsky, '57). Many of the data to be presented were obtained from the lyophilized samples used during the study of pancreatic enzyme secretion. Nitrogen analyses were made using an all-glass, semi-micro distillation unit following 5 to 6 hours digestion with sulfuric acid and copper sulfate as the catalyst. The addition of three or 4 drops of hydrogen peroxide with a final half hour refluxing terminated the digestion.

RESULTS AND DISCUSSION

Carroll, Hensley and Graham ('52) had observed that net unabsorbed nitrogen in the small intestine of rats fed raw soybean was higher than in rats fed the heated meal. However, Borchers ('53) was unable to repeat their results. No obvious explanation for the discrepancy was apparent. The data in table 1 show that the unabsorbed nitrogen and the percentage of nitrogen in the small intestine of rats fed raw soybean were significantly higher than in the animals fed

TABLE 1

Nitrogen in the intestinal contents of rats fed raw and heated soybean meal diets

HOURS POST-FEEDING	RAW SOYBEAN MEAL			HEATED SOYBEAN MEAL		
	No. of animals	Nitrogen [1]	Nitrogen in contents [2]	No. of animals	Nitrogen	Nitrogen in contents
		%	*mg*		%	*mg*
0	6	8.00 ± 0.29 [3]	25.8 ± 2.7	6	5.37 ± 0.24	22.9 ± 2.72
3	8	7.63 ± 0.11	34.8 ± 2.4	8	5.01 ± 0.21	19.6 ± 1.62
6	8	6.75 ± 0.20	28.2 ± 2.4	8	4.72 ± 0.16	20.6 ± 1.53

[1] Significantly higher ($p < 0.01$) than the heated soybean group (Snedecor, '48).

[2] Significantly higher at three hours ($p < 0.01$) and 6 hours ($p < 0.03$) than the heated soybean group.

[3] Standard error of the mean $= \sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

heated soybean. This difference was not related to the total amount of intestinal contents present in the rats fed raw soybean, since the range of the contents at all intervals for both groups was between 0.400 and 0.456 gm. A comparison of the relative food intakes of the two groups on the day of an experiment, representing 28 and 22 animals, respectively, showed that 7.4 ± 0.55 gm of raw soybean diet were consumed while 10.3 ± 0.43 gm of the heated protein diet were eaten per rat. Therefore, not only was less nitrogen ingested by the rats fed the raw soybean diet, but in addition, appreciable amounts were apparently not utilized by the animal. The excess nitrogen could be caused by an inability to hydrolyze

and absorb some nitrogen-rich complex in the raw soybean protein, or, alternatively, be provided by the increased pancreatic secretions evoked by the trypsin inhibitor.

In order to determine whether the nitrogen might be due to an unabsorbed complex in the unheated soybean protein, trypsin inhibitor concentrate and crystalline soybean antitrypsin were added to basic heated soybean diets at 2 and 0.5% respectively. At these levels, a pancreatic enzyme discharge was induced. Table 2 shows that both the percentage and the total nitrogen in the small intestine increased and remained as high with 0.5% of the crystalline inhibitor as with 2% of the crude inhibitor preparation. At a concentration of 0.5% in the diet, the maximum amount of nitrogen contributed by the crystalline inhibitor would be less than 1 mg for every gram of diet passed into the intestine. Since the high level of intestinal nitrogen was maintained for an extended period of time, it would appear unlikely that the dietary factor provided it. Also, the intestinal nitrogen was highest at the time when greater than normal intestinal trypsin activity existed, so an inability to hydrolyze the soybean protein could not explain the elevated nitrogen. No evidence of delayed digestion of the raw soybean diet was detected when the rate of removal of solids from the stomach was compared with that from animals on the heated soybean diet.

The pancreas, following stimulation by trypsin inhibitor, had been shown to reflect consistently the high enzyme activities in the intestine by showing a reduction in the residual enzyme activity (Lyman and Lepkovsky, '57). Table 3 indicates that a similar effect was obtained when residual pancreatic nitrogen was determined following stimulation. During the fasting period, pancreatic nitrogen was at its highest level and similar for both groups of animals. However, immediately after eating (zero hour), the nitrogen in the group fed the raw soybean decreased (with the accompanying loss of enzyme activity), recovered somewhat by three hours and remained nearly constant during 6 hours post-feeding. This

TABLE 2

TABLE 2

The effect of trypsin inhibitor in various diets on unabsorbed intestinal nitrogen [1]

HOURS POST-FEEDING	TRYPSIN INHIBITOR CONCENTRATE WITH HEATED SOYBEAN MEAL			0.5% CRYSTALLINE TRYPSIN INHIBITOR WITH HEATED SOYBEAN MEAL			BASIC HEATED SOYBEAN MEAL		
	Nitrogen in contents		Trypsin activity [2]	Nitrogen in contents		Trypsin activity [2]	Nitrogen in contents		Trypsin activity [2]
	%	*mg*		%	*mg*		%	*mg*	
0	7.38	31.8	0.225	7.24	30.1	0.553	5.00	24.8	0.437
3	6.61	31.2	0.450	7.00	33.0	0.561	4.63	20.8	0.333
6	6.02	28.1	0.755	6.10	27.0	0.624	4.53	18.4	0.358

[1] Each set of numbers represents the average value from two rats at the periods indicated.
[2] Milliequivalents of tyrosine/10 minutes incubation.

TABLE 3

The effect of raw (R.S.) and heated soybean meals (H.S.) on the dry weight, per cent and total nitrogen of the whole lyophilized pancreas

HOURS POST-FEEDING	NO. OF ANIMALS IN EACH GROUP	DRY WT. PER 100 GM BODY WEIGHT [1]		TOTAL NITROGEN IN PER CENT		TOTAL NITROGEN PER 100 GM BODY WEIGHT [1]	
		R.S.	H.S.	R.S.	H.S.	R.S.	H.S.
		mg	*mg*			*mg*	*mg*
F	4	137 ± 6 [2]	141 ± 3	12.6 ± 0.2	13.3 ± 0.2	17.8 ± 0.8	18.6 ± 0.4
0	7	88 ± 2	113 ± 10	13.2 ± 0.1	12.9 ± 0.2	11.6 ± 0.3	14.6 ± 1.2
3	9	106 ± 4	124 ± 5	12.5 ± 0.2	12.6 ± 0.2	13.2 ± 0.5	15.6 ± 0.6
6	9	103 ± 4	120 ± 6	12.9 ± 0.2	13.0 ± 0.1	13.4 ± 0.6	15.7 ± 0.9

[1] When the data for the nitrogen and dry weight of the pancreas from the two groups of rats were tested for significance, a significant difference between R.S. and H.S. existed at 0 hour ($p < 0.05$) 3 hours ($p < 0.02$) and 6 hours ($p < 0.05$) after eating.
[2] Mean ± standard error.

depletion of nitrogen from the pancreas at zero, three and 6 hours following consumption of the raw soybean diet was significantly greater than the depletion noted in the heated soybean group. The nitrogen, calculated as percentage, showed no change in either group of rats at the various periods. Since the pancreas size decreased following the stimulation, other components of the pancreatic tissue appeared to have been depleted along with the enzymes discharged.

TABLE 4

The effect of raw and heated soybean meal on the nitrogen and amylase, lipase and trypsin activity in the intestine, cecum and colon of the rat [1]

SAMPLE	INTESTINAL CONTENTS		NITROGEN		TOTAL NITROGEN		AMYLASE [2]		LIPASE [3]		TRYPSIN [4]	
	R.S.	H.S.	R.S.	H.S.	R.S.	H.S.	R.S.	H.S.	R.S.	H.S.	R.S.	H.S.
	gm	*gm*	%	%	*mg*	*mg*						
Intest.	0.39	0.37	6.8	5.0	26.6	18.6	33.3	6.8	48.5	5.0	0.72	0.31
Cecum	0.41	0.46	9.0	4.8	36.8	21.8	28.8	0.2	37.1	0.8	0.45	0.06
Colon	0.20	0.29	7.2	4.1	14.4	11.8	11.2	0.1	20.4	0.3	0.18	0.02

[1] All values represent the average of separate analyses from two rats at the 3-hour and 6-hour periods. The results from the 0-hour period were omitted because very little of the enzyme had reached the cecum or colon so soon after eating.

[2] Amylase units $\times 10^{-3}$.

[3] Milliequivalents of acid/hr.

[4] Milliequivalents of tyrosine/10 min. incubation.

If one assumes that excess nitrogen depleted from the pancreas in the rats fed raw soybean represents protein secreted into the small intestine in the form of newly synthesized enzymes, the prolonged nature of the discharge could result in a substantial loss of nitrogen to the rats, if not utilized. Therefore, the concentration of nitrogen along the digestive tract was investigated by analyzing the contents from the small intestine, cecum, and colon for nitrogen, amylase, lipase, and trypsin activity. The results are presented in table 4. The percentage of intestinal nitrogen in the animals fed raw soybean remained high throughout the digestive tract when compared with that of rats fed the heated soybean diet. Also, the total amount of unabsorbed nitrogen

in the raw soybean animals was elevated in the intestine and cecum, but declined in the colon. All three of the enzymes from the raw soybean group remained active in the cecum and the colon, indicating that a good share of the enzyme protein was intact and unabsorbed. In contrast to this was the almost complete loss of enzyme activity in the cecum and colon of rats fed the autoclaved soybean diet. The enzyme destruction might have been caused by bacterial action or the formation of inactive complexes. However, the inactivation was so nearly complete, it almost suggests a physiological mechanism designed to protect the organism from self-digestion. The abnormally high secretion of enzymes induced by the soybean inhibitor appeared to interfere effectively with whatever mechanism inactivated the normally secreted enzymes.

Although Carroll et al. ('52) had reported more net unabsorbed nitrogen in the intestine of rats fed raw soybean, the net digestibility of the raw and heated protein, as determined from fecal nitrogen, was similar. It was proposed that much of the nitrogen that escaped absorption in the small intestine must later be absorbed from the cecum or colon. Because of bacterial degradation of the amino acids, this nitrogen would, presumably, have little utility for growth. Several workers had previously noticed the similarity in digestibility of raw and heated soybean protein, whereas the biological value was much in favor of the heated protein (Johnson et al., '39; Desikachar and De, '47; Melnick, Oser and Weiss, '46). If the results of others are applicable to those presented in this investigation, a part of the excess nitrogen in the rats fed raw soybean would have to be absorbed from the colon, since the evidence does not indicate any loss of nitrogen from the cecum. Unfortunately, exact interpretation of the data is made difficult because loss of colonic material prior to autopsy could have contributed to the reduced amount of nitrogen in both groups of animals.

The data suggest that the high concentration of intestinal nitrogen in the rats fed raw soybean could have been caused

by the stimulated secretory response of the pancreas. Daly and Mirsky ('52) concluded that the mouse pancreas may lose as much as 90% of its protein in the form of discharged enzymes when sufficiently stimulated. However, they were unable to demonstrate a loss in weight of the pancreas during the period of the enzyme discharge. Magee and Anderson ('55) postulated that the depressed growth exhibited by rats injected with urecholine for 21 days was due to nitrogen wasted in the form of excessive enzyme secretions. Unpublished data from this laboratory had shown that rats, while being fed raw soybean diets, excreted high levels of proteolytic activity in their feces for many weeks. Therefore, if one accepts the view that the soybean antitrypsin plays an important part in the growth depression in rats produced by unheated soybean protein, it would appear that any inhibiting action may be exerted through a loss of essential amino acids from endogenous sources rather than through depression of normal intestinal protein hydrolysis.

It is conceivable that the excess cystine observed by Carroll et al. ('53) in the contents of the small intestine of rats fed raw soybean originated from the pancreatic secretions. A loss of cystine by this means could reasonably be expected to produce a deficiency of methionine through a demand for this amino acid to meet the increased cystine requirements for protein (enzyme) synthesis.

SUMMARY

Rats fed raw soybean diets had a higher concentration of net unabsorbed nitrogen in the intestine than did rats fed the heated protein meal. The increased nitrogen in the intestine was accompanied by a correspondingly greater depletion of pancreas nitrogen. Soybean trypsin inhibitor at 0.5% of a heated soybean diet (a level capable of evoking a pancreatic enzyme response) also increased the intestinal nitrogen. The elevated level of nitrogen could not be accounted for by the undigested inhibitor or by a failure of intestinal

proteolysis. The nitrogen concentration remained high in the small intestine and cecum, but was reduced in the colon. Amylase, trypsin and lipase activity remained increased throughout the intestinal tract of animals fed raw soybean, whereas rats fed the heated meal showed almost complete destruction of the enzymes in the cecum and colon. As a result, it was suggested that the high intestinal nitrogen originated from the stimulated pancreatic secretions. A possible relation of these observations to the apparent poor utilization of unheated soybean protein was mentioned.

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BENEFICIAL EFFECTS OF ALFALFA AND OTHER SUCCULENT PLANTS ON THE GROWTH OF IMMATURE GUINEA PIGS FED A MINERALIZED DRIED MILK RATION[1]

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Available data indicate that alfalfa and other succulent plants contain a factor (or factors), apparently distinct from any of the known nutrients, which is essential for optimal nutrition in animals fed a mineralized milk ration. As early as 1934 Elvehjem and co-workers found that milk produced by cows on a regular winter ration was inferior in nutritive value to milk produced by cows that had access to summer pastures. Immature rats fed mineralized summer milk grew more than 4 gm per day over a 6-week period in contrast to a weight increment of only 2.5 gm per day for rats fed a mineralized winter milk. The feeding of 3 ml of grass juice per day to rats on mineralized winter milk, however, stimulated growth to more than 4 gm per day, a rate comparable to that obtained on summer milk (Kohler et al., '36). Since guinea pigs are herbivorous it was felt by Kohler et al. ('38) that this species might be a more suitable animal for the assay of a "grass juice factor" than the rat which is carnivorous. Subsequent findings by these workers demonstrated that guinea pigs lost weight, developed a bloated unthrifty appear-

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ance and failed to survive when fed a mineralized winter-milk ration supplemented with orange juice as a source of vitamin C. These effects were completely counteracted by the concurrent feeding of 20 ml of fresh grass juice per day (Kohler et al., '38). Studies on the distribution of the "grass juice factor" indicated that dehydrated cereal grass, rye grass, alfalfa, young white clover, peas, pea shells, cabbage, turnip tops, lettuce and oats were all good sources of this factor. It was also observed that the activity of these materials varied with the stage of growth, the mature plants being much less effective in general than rapidly growing ones (Kohler et al., '38; Randle et al., '40). These findings suggested that the seasonal change in the nutritive value of milk was related to the presence or absence of a factor (or factors) in the forage ingested by the cows. It would appear that the green succulent grass consumed during certain periods of the summer contained an amount of the "grass juice factor" which was sufficient, not only for the body needs, but also for transmission into the milk. In contrast, during the winter months the cow ingested mainly dried fodder which had lost much of its potency in respect to this factor.

Developments in the science of nutrition and improvements in the feeding practices of dairymen have served to improve the nutritive value of winter milk to the extent that the differences noted by Elvehjem, Kohler and others as to the comparative nutritive value of mineralized winter and summer milk are not readily demonstrable with milk samples obtained under present-day conditions. In the present communication, however, data are presented indicating that dried alfalfa and other succulent plants also promote growth in guinea pigs fed a ration containing mineralized spray-process whole milk powder. The protective factor (or factors) is apparently distinct from any of the known nutrients.

PROCEDURE

A series of experiments was designed to study the effects on growth and survival of guinea pigs of the addition of

alfalfa and other succulent plants to a mineralized milk ration. The basal diet for the first 4 experiments consisted of spray-process whole-milk powder,[2] 85%; cellulose,[3], 10% and agar,[4] 5%. To each kilogram of the above diet were added 5000 U.S.P. units of vitamin A, 500 U.S.P. units of vitamin D_2 and the following mineral salts: ferric pyropyhophate, 425 mg; copper sulfate, 20 mg; and manganese sulfate, 15 mg. The supplements were added in place of an equal amount of milk powder. Each guinea pig also received 6 times weekly an oral supplement of 10 mg ascorbic acid in 0.1 ml water and twice weekly an oral supplement of 15 mg alpha-tocopherol acetate in 0.1 ml of 95% ethyl alcohol. In the preparation of the diet, the agar was first mixed with warm water in the proportion of 300 ml of water to each 50 gm of agar. To the water-agar mixture were then added the remaining components of the diet. After thorough mixing the diet was passed through an electric meat grinder and the resulting macaroni-like strands placed in shallow pans and dried in an oven at a temperature of 110 to 120°F. The alfalfa meal and other materials to be tested were added to the basal ration in place of equal amounts of whole-milk powder (table 1). With the exception of the stock ration,[5] which was purchased from a local distributor, all diets were made up bi-weekly and stored under refrigeration when not in use. The guinea pigs were housed in large metal cages with raised screen bottoms (3 to 5 animals per cage) and were provided with water and food ad libitum. The animals were fed daily and all food not consumed 24 hours after feeding was discarded. The feeding was continued for 6 or 12 weeks (see table 1) or until death, whichever occurred first.

[2] Challenge Spray-Process Powdered Whole Milk, Challenge Cream and Butter Assn., Los Angeles, California.

[3] Solka-floc BW 200, Brown and Co., Berlin, New Hampshire.

[4] Agar Agar U.S.P. Kobe no. 1 Flakes, Hathaway Allied Products, Los Angeles, California.

[5] Purina Rabbit Chow Checkers WO, Ralston Purina Co., St. Louis, Missouri. Each guinea pig fed this diet also received 6 times weekly an oral supplement of 10 mg ascorbic acid.

TABLE 1

Comparative effects of alfalfa meal and other supplements on the weight increment of immature female guinea pigs fed a mineralized dried milk ration [1,2,3,4]

SUPPLEMENTS ADDED TO BASAL RATION	INITIAL BODY WT.	AVERAGE GAIN IN BODY WEIGHT AFTER THE FOLLOWING WEEKS OF FEEDING		
		4th	6th	12th
	gm	gm	gm	gm
Experiment 1				
None	274	50 (12)	72 (9)	184 (9)
Lettuce	272	49 (13)	74 (12)	178 (9)
Oven-dried alfalfa meal,[5] 20%	269	179 (15)	270 (15)	406 (13)
Sun-dried alfalfa meal,[5] 20%	267	156 (14)	239 (14)	386 (14)
Vaccum-dried alfalfa meal,[5] 20%	266	159 (15)	215 (15)	347 (14)
(Stock ration)	271	141 (15)	201 (14)	360 (14)
Experiment 2 (a)				
		4th	6th	
None	241	50 (12)	91 ± 14.3 (12)	
Aureomycin HCl[6]	243	57 (7)	110 ± 16.1 (7)	
B vitamins, C and K[7]	236	83 (10)	122 ± 7.3 (10)	
Vitamins A, D and E[8]	237	68 (9)	114 ± 17.9 (9)	
Casein,[9] 10%	239	62 (12)	104 ± 12.4 (12)	
Corn oil, 5%	241	32 (10)	97 ± 11.2 (10)	
Cellulose,[10] 5%	241	55 (10)	125 ± 14.4 (9)	
Alfalfa ash, 2.5%	239	76 (11)	134 ± 11.8 (11)	
Potassium acetate, 2.5% and magnesium oxide, 0.5%	240	82 (12)	141 ± 12.9 (12)	
Sun-dried alfalfa meal, 20%	240	173 (12)	253 ± 16.0 (12)	
Alfalfa residue,[11] 12%	238	126 (12)	182 ± 14.2 (12)	
Dried alfalfa juice, 8%	239	109 (12)	163 ± 10.8 (10)	
Experiment 2 (b)				
None	266	56 (10)	72 ± 12.3 (9)	
Oven-dried alfalfa meal, 20%	254	162 (12)	256 ± 14.8 (12)	
Combined supplements[12]	263	91 (12)	122 ± 16.2 (12)	
Tuna meal, 4%	262	68 (11)	116 ± 12.1 (10)	
Tuna solubles, 5%	261	112 (12)	153 ± 12.9 (11)	
Arginine HCl + choline[13]	262	64 (10)	119 ± 16.1 (10)	
Experiment 3				
None	229	65 (9)	76 (8)	
Oven-dried alfalfa lot #1, 5%	229	100 (12)	141 (9)	
Oven-dried alfalfa lot #1, 10%	227	144 (12)	194 (11)	
Oven-dried alfalfa lot #1, 20%	226	171 (12)	237 (12)	
Oven-dried alfalfa lot #2, 20%	225	159 (12)	207 (11)	
Oven-dried alfalfa lot #3, 20%	228	195 (12)	253 (12)	
Dehydrated rye grass, 20%	229	165 (12)	243 (11)	
Dehydrated orchard grass, 20%	228	168 (12)	221 (11)	
Dehydrated wheat grass, 20%	229	135 (9)	285 (4)	
Dehydrated fescue grass, 20%	227	121 (12)	186 (11)	

TABLE 1 *(continued)*

SUPPLEMENTS ADDED TO BASAL RATION	INITIAL BODY WT.	AVERAGE GAIN IN BODY WEIGHT AFTER THE FOLLOWING WEEKS OF FEEDING		
		4th	6th	12th
	gm	*gm*	*gm*	*gm*
Dehydrated oat grass, 20%	229	162 (12)	226 (10)	
Alfalfa residue, 12%	227	136 (12)	185 (10)	
Alfalfa juice concentrate, 50% solids (lot #FD-11-115D), 5%	229	73 (11)	135 (9)	
Dried alfalfa juice concentrate, (lot #FD-11-115D), spray dried, 2.5%	228	101 (12)	162 (11)	
Alfalfa juice concentrate treated with copper, 50% solids (lot #FB-12-24B), 5%	227	80 (9)	146 (8)	
Dried alfalfa juice concentrate treated with copper (lot #FB-12-24B), spray dried, 2.5%	229	103 (11)	153 (10)	

[1] The values within parentheses indicate the number of animals which survived and on which averages are based. Fifteen animals per group were employed in experiment 1; 12 in experiments 2 and 3.

[2] The duration of experiment 1 was 12 weeks; experiments 2 and 3, 6 weeks.

[3] The standard error of the mean is included with the 6-week weight increment for animals in experiment 2. The standard error of the mean was calculated as follows: $\frac{\sqrt{\frac{\Sigma d^2}{n}}}{\sqrt{n}}$ where "d" is the deviation from the mean and "n" is the number of observations.

[4] The alfalfa samples were kindly provided by Dr. S. Tenkoff of Nutrilite Products, Inc., Buena Park, California. The alfalfa juice concentrates were supplied by Dr. George O. Kohler of the Cerophyl Laboratories, Kansas City, Kansas. The dehydrated rye grass, orchard grass, wheat grass, fescue grass and oat grass were obtained from the National Chlorophyll and Chemical Company, Lamar, Colorado. The tuna meal and tuna solubles (50% solids) were kindly provided by Dr. E. Geiger of the Van Camp Sea Foods Company, Terminal Island, California.

[5] The oven-dried, sun-dried and vacuum-dried alfalfa were all prepared from the same batch of freshly cut alfalfa.

[6] One hundred milligrams aureomycin HCl per kilogram of diet.

[7] The following vitamins were added per kilogram of diet: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; vitamin B_{12}, 150 μg; and 2-methyl-naphthoquinone, 5 mg.

[8] Five thousand U.S.P. units of vitamin A and 500 U.S.P. units of vitamin D_3 per kilogram of ration plus an oral supplement of 15 mg alpha-tocopherol acetate in 0.1 ml of 95% ethyl alcohol provided 4 times weekly to each guinea pig.

[9] Vitamin-free Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[10] Solka-floc BW 200, The Brown Company, Berlin, New Hampshire.

[11] The water-washed alfalfa pulp remaining after the extraction of the juice.

[12] Alfalfa ash, 2.5%; casein, 10%; corn oil, 5%; cellulose, 5%; and the vitamin supplements indicated in footnotes 7 and 8.

[13] Arginine·HCl, 0.5% and choline chloride, 0.2%.

RESULTS

Experiment 1

Beneficial effects of alfalfa meal. Ninety female guinea pigs averaging 270 gm in body weight (range 237 to 331 gm) were selected for this experiment. The animals were divided into 6 comparable groups of 15 guinea pigs each and were fed the basal diet alone or the basal diet with supplements as indicated in table 1. The findings indicate that supplements of dried alfalfa meal significantly increased the weight increment of immature guinea pigs fed a mineralized dried milk ration. All three alfalfa supplements tested (the oven-dried, sun-dried and vacuum-dried meals) had marked activity. Of the 15 guinea pigs fed the basal ration, 9 survived the experimental period of 12 weeks with an average weight increment of 184 gm. The animals in this group exhibited an unthrifty appearance, ruffled fur, varying degrees of alopecia and a bloated "pot-bellied" appearance. Supplements of fresh lettuce when added to the basal ration were without significant effect on either growth, survival or appearance. Supplements of dried alfalfa meal, however, when added to the basal ration resulted in a weight increment approximately twice that of animals fed the basal ration. Guinea pigs fed the alfalfa-containing diets appeared normal in all respects; their fur was smooth and sleek; they survived the 12-week experimental period with virtually no casualties and were indistinguishable both in weight and appearance from animals fed the stock ration.

Surviving animals were autopsied after 12 weeks of feeding and a routine histological examination was made of the tissues of guinea pigs in the various groups. No pathological findings were observed with the exception of a mild fatty infiltration of the liver of animals fed the basal ration or the basal ration plus lettuce. Significant differences were observed, however, in respect to ovarian and uterine weights. The average ovarian weight of guinea pigs fed the basal ration and the basal ration plus lettuce was 59.7 mg (range 39 to 98 mg)

in contrast to an average ovarian weight of 128 mg (range 94 to 182 mg) for animals fed the alfalfa supplements or the stock ration. In the series fed the basal ration and the basal ration plus lettuce the uteri averaged 223.3 mg (range 134 to 386 mg) in contrast to an average weight of 1424 mg (range 754 to 2302 mg) for animals fed the alfalfa supplements or stock ration. No significant differences in ovarian or uterine weights were observed between guinea pigs fed the various alfalfa supplements or between these animals and those on the stock ration. In preliminary studies with 6 male guinea pigs (three on the basal ration and three on the basal ration plus 20% oven-dried alfalfa), findings were obtained which were comparable to those reported for female guinea pigs on the same diets. Animals were selected at a body weight between 260 and 290 gm. After 12 weeks of feeding the average weight increments were 238 and 548 gm respectively for male guinea pigs on the basal ration and basal ration plus alfalfa with seminal vesicle weights averaging 800 mg and 2910 mg respectively for the two series.

Experiment 2

Comparative effects of dried alfalfa, alfalfa fractions and supplements of known nutrients. (a) One hundred and forty-four female guinea pigs averaging 242 gm in body weight (range 204 to 283 gm) were selected for this experiment. The animals were divided into 12 comparable groups of 12 guinea pigs each. One group was fed the basal ration; the remaining groups were fed diets consisting of the basal ration plus the supplements listed in table 1. In agreement with the observations made in experiment 1, the addition of 20% sun-dried alfalfa meal to the basal ration resulted in a significant increment in body weight. Both the dried alfalfa juice and the water-washed alfalfa pulp remaining after the extraction of the juice also showed significant growth-promoting activity although the weight increment obtained with these alfalfa fractions was appreciably less than that obtained with the

whole alfalfa meal. In contrast to the results obtained above, supplements of all the known vitamins, aureomycin HCl, fat in the form of corn oil or protein in the form of casein had little if any growth-promoting effect. A slight increment in body weight over that obtained with the latter supplements was observed in guinea pigs fed an increased amount of cellulose, alfalfa ash at a level equivalent to the ash content of the 20% alfalfa meal supplement, or supplements of potassium acetate and magnesium oxide. The increments in body weight obtained with these supplements, however, were significantly smaller than that obtained with alfalfa meal.

(b) Seventy-two female guinea pigs averaging 262 gm in body weight (range 212 to 304 gm) were next selected for the following experiment. Animals were divided into 6 comparable groups of 12 guinea pigs each. One group was fed the basal ration; the remaining groups were fed diets consisting of the basal ration plus the supplements listed in table 1. The experimental procedure was similar to that employed in experiment 2a except that the guinea pigs fed the basal ration and the basal ration plus alfalfa meal were kept in individual metal cages. Food consumption was determined daily for all animals in the latter groups from the 8th through the 28th day of the experiment. In agreement with earlier findings the addition of 20% oven-dried alfalfa meal to the basal ration resulted in a significant increment in body weight. The tuna solubles supplement also resulted in a significant weight increment although less than that obtained with the alfalfa meal. The weight increment of guinea pigs fed the other supplements was slightly but not significantly better than that of animals on the basal ration.

The above findings suggest that in addition to its content of ash constituents, roughage, vitamins, arginine, choline, protein or fat, alfalfa meal contains a factor or factors apparently distinct from any of the known nutrients which promoted a significant increment in body weight of immature guinea pigs fed a mineralized spray-process dried milk ration.

The possibility that the growth-promoting effects of alfalfa meal may have been due to a particular amino acid balance rather than to the total protein or amino acid content *per se* of this supplement has not, however, been thoroughly excluded although it appears unlikely due to the small growth response obtained with such protein-containing supplements as casein and tuna meal.

The ad libitum food consumption of guinea pigs fed the alfalfa-containing diet was significantly greater than that of animals on the basal ration. The average food intake of animals on the alfalfa diet from the 8th through the 28th day of the experiment was 23.8 gm per guinea pig per day in contrast to an average food intake of 11.4 gm per day for animals on the basal ration. This raises the question as to what extent differences in growth on the two rations may have been due to differences in palatability of the two diets. That some factor other than differences in palatability is involved, however, is indicated by the fact that animals on the basal ration not only were smaller but exhibited varying degrees of alopecia, a distended (bloated) abdomen, and an unthrifty appearance in contrast to the smooth sleek fur and normal appearance of animals fed the alfalfa-containing diet, differences that could not be accounted for on the basis of a reduced food intake *per se*.

Experiment 3

Comparative effects of dehydrated alfalfa, rye grass and other succulent plants. One hundred and ninety-two female guinea pigs averaging 228 gm in body weight (range 202 to 274 gm) were employed in the present experiment. Animals were divided into 16 comparable groups of 12 guinea pigs each. One group was fed the basal ration; the remaining groups were fed diets consisting of the basal ration plus the various supplements listed in table 1. In agreement with earlier findings oven-dried alfalfa, when incorporated at a 20% level in the basal ration, resulted in a significant increment in body weight.

Considerable variation was observed, however, in the growth-promoting activity of different batches of alfalfa. Dehydrated rye grass, orchard grass, wheat grass, fescue grass and oat grass when fed at a 20% level in the diet also showed significant growth-promoting activity. Oven-dried alfalfa when fed at 5% and 10% levels in the diet resulted in a significant increment in body weight over that obtained on the basal ration but less than that obtained with the 20% level of supplementation. A high mortality occurred among guinea pigs fed the wheat grass supplement which did not occur among animals fed the other supplements. Surviving animals in this group, however, were among the largest observed in the experiment. In agreement with earlier findings alfalfa residue when fed at a 12% level in the diet showed significant growth-promoting activity although less than that obtained with 20% whole alfalfa. Four samples of alfalfa juice concentrate incorporated in the basal ration at a 2.5% level on a dry weight basis also resulted in a significant increment in body weight over that obtained on the basal ration but less than that obtained with the alfalfa residue.

Experiment 4

Effects of physical state of the diet and "processing procedures." The following experiment was undertaken to determine to what extent the physical state of the diet and "processing procedures" (mixing the various ingredients with wet agar, grinding the mass and drying it) may have impaired the nutritive value of the basal ration by promoting such possible effects as destruction or inactivation of essential nutrients, decreased digestibility of proteins, etc. Forty-eight female guinea pigs averaging 239 gm in body weight (range 197 to 268 gm) were divided into 4 comparable groups of 12 animals each and were fed the following rations ad libitum: (A) basal ration (B) basal ration plus 20% oven-dried alfalfa meal (C) basal ration in powdered form and (D) basal ration plus 20% oven-dried alfalfa meal in powdered

form. Diets C and D were identical to rations A and B respectively with the exception that the various ingredients were mixed into the diet without the addition of water, grinding and subsequent drying. Any possible impairment of nutritive value that may have occurred as a consequence of such processing procedures was thus eliminated in rations C and D. The experimental procedure was similar to that employed in earlier experiments. Feeding was continued for 6 weeks.

TABLE 2

Comparative effects of powdered and "pelleted" rations on the weight increment of immature guinea pigs fed a mineralized dried milk ration

(12 animals per group)[1]

DIETARY GROUP [2]	INITIAL BODY WT.	AVERAGE GAIN IN BODY WEIGHT AFTER THE FOLLOWING WEEKS OF FEEDING	
		4th	6th [3]
	gm	*gm*	*gm*
A	238	76 (11)	92 ± 15.1 (9)
B	236	144 (12)	210 ± 12.4 (11)
C	241	60 (9)	78 ± 14.6 (8)
D	241	118 (12)	178 ± 14.2 (10)

[1] The values within parentheses indicate the number of animals which survived and on which averages are based.

[2] A, basal ration; B, basal ration plus 20% oven-dried alfalfa meal; C, basal ration in powdered form; and D, basal ration plus 20% oven-dried alfalfa meal in powdered form.

The results are summarized in table 2. In agreement with earlier findings the addition of alfalfa meal to the basal ration resulted in a significant increment in body weight. The increased growth occurred on both the pelleted and powdered rations (diets B and D). The findings indicate further that the weight increment of guinea pigs fed the pelleted rations (diets A and B) was not inferior to that of animals fed the same rations in powdered form (diets C and D). If the nutritive value of diets A and B had been impaired as a result of "processing procedures," one would have anticipated that the weight increment of animals on these diets would

have been less than that of animals fed the unprocessed rations. It would appear, therefore, that "processing procedures" in the preparation of the basal pelleted ration were not responsible for its poor nutritive value. On the contrary, the weight increment of guinea pigs fed the pelleted diets was approximately 20% greater than that of animals fed similar diets in powder form.

Experiment 5

Effects of alfalfa meal supplementation on the weight increment of guinea pigs fed purified rations. The data obtained in experiments 1 to 4 indicate that supplements of alfalfa meal stimulated a significant increment in body weight in guinea pigs fed a mineralized dried-milk ration. The following experiment was undertaken to determine whether such supplements would have a similar effect in guinea pigs fed a semi-synthetic ration. The purified rations employed in these studies were similar to diet no. 13 of Reid and Briggs ('53) but differed in their roughage components. In preliminary investigations it was observed that when guinea pigs were transferred to purified rations at a body weight of 200 to 250 gm, the number of animals surviving and the average weight increment of the survivors was appreciably greater on diets containing 5% agar [6] than when the roughage of the diet was provided in other forms.[7,8] Hence agar was incorporated at a 5% level in all purified diets in the present series. Studies were conducted with two types of rations. In one dietary, carbohydrate was provided as a combination of sucrose, corn starch and dextrose; the other contained lactose as the principal carbohydrate of the ration. The experiments with lactose were undertaken to determine whether the poor growth of guinea pigs on the mineralized dried-milk ration might not be due to the high lactose content of this

[6] See footnote 4, page 297.

[7] See footnote 6, page 297.

[8] Cellophane Spangles, obtained from the Rayon Processing Company of Pawtucket, R.I.

diet. It was felt that the growth-promoting effects of alfalfa meal when added to such a ration might possibly be due to a factor(s) in alfalfa which counteracted the growth-retarding effects of lactose when the latter was ingested in excessive

TABLE 3

Composition of semi-synthetic experimental diets

INGREDIENT [1]	DIET 101	DIET 102	DIET 103	DIET 104
	gm	gm	gm	gm
Choline chloride	2	2	2	2
Inositol	2	2	2	2
Magnesium oxide	5	5	5	5
Potassium acetate	25	25	25	25
Cottonseed oil	50	50	50	50
Agar	50	50	50	50
Salt mixture [2]	60	60	60	60
Cellulose [3]	100	100	100	100
Sucrose	100	50	37.5	
Dextrose	106	56	37.5	
Cornstarch	200	150	81	6
Casein [4]	300	300	300	300
Lactose			250	250
Oven-dried alfalfa meal		150		150

[1] To each kilogram of the above diets were added the following vitamins: thiamine hydrochloride, 16 mg; riboflavin, 16 mg; pyridoxine hydrochloride, 16 mg; calcium pantothenate, 40 mg; nicotinic acid, 200 mg; biotin, 1 mg; folic acid, 10 mg; vitamin B_{12}, 100 μg; 2-methyl-naphthoquinone, 5 mg; para-aminobenzoic acid, 100 mg; vitamin A, 5000 U.S.P. units; vitamin D_2, 500 U.S.P. units; and alpha-tocopherol acetate, 100 mg. The vitamins were added in place of an equal amount of corn starch. Each guinea pig also received 6 times weekly an oral supplement of 10 mg ascorbic acid.

[2] Hubbell, Mendel and Wakeman Salt Mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Solka-floc BW 200, The Brown Company, Berlin, New Hampshire.

[4] Vitamin-free Test Casein. General Biochemicals, Inc., Chagrin Falls, Ohio.

amounts. If such were the case then supplements of alfalfa meal might also be expected to exert a growth-promoting effect in guinea pigs fed a semi-synthetic ration of similar lactose content.

Forty-eight female guinea pigs averaging 234 gm in body weight (range 210 to 256 gm) were divided into 4 comparable

groups of 12 animals each and were fed the 4 diets shown in table 3. All diets were made weekly and stored under refrigeration when not in use. Animals were placed in metal cages with raised screen bottoms (three animals per cage) and were provided food and water ad libitum. Animals were fed daily, and all food not consumed 24 hours after feeding was discarded. Feeding was continued for 28 days. With the exception of one guinea pig each on diets 101 and 104, all animals survived the experimental period. The average weight increment of guinea pigs on the various diets was as follows: diet 101, 109 ± 10.3 gm;[9] diet 102, 122 ± 10.6 gm; diet 103, 116 ± 9.8 gm; and diet 104, 99.8 ± 8.7 gm. Differences in weight increment between animals in the various groups were not statistically significant. The guinea pigs in all 4 groups appeared normal in all respects; their fur was smooth and sleek; and they were indistinguishable in appearance from animals of a comparable age that had been fed a stock ration.[10] The findings indicate that supplements of alfalfa meal did not promote a significant increment in body weight in guinea pigs fed semi-synthetic rations. These results are in contrast to those obtained with an identical supplement when fed to guinea pigs on a mineralized dried-milk ration. The findings also indicate that the ingestion by guinea pigs of a purified diet containing 25% lactose did not result in growth retardation, diarrhea or other deleterious effects commonly associated with an excessive lactose intake.

DISCUSSION

The findings indicate that supplements of desiccated whole alfalfa promoted a significant increment in body weight in immature guinea pigs fed a mineralized spray-process dried-milk ration. Both the dried alfalfa juice and the water-washed alfalfa pulp remaining after the extraction of the juice also showed significant growth-promoting activity. In

[9] Including standard error of the mean. See footnote 3, table 1.

[10] See footnote 5, page 297.

contrast to the results obtained with these materials, supplements of all the known vitamins, arginine, choline, aureomycin HCl, fat in the form of corn oil or protein in the form of casein or tuna meal had little if any growth-promoting effect. A slight increment in body weight over that obtained with the latter supplements occurred in guinea pigs fed an increased amount of cellulose, alfalfa ash at a level equivalent to the ash content of the alfalfa meal supplement, or supplements of potassium acetate and magnesium oxide. The increments in body weight obtained with these supplements, however, were significantly smaller than that obtained with alfalfa meal. These findings suggest that in addition to its content of ash constituents, roughage, vitamins, arginine, choline, protein and fat, alfalfa meal contains a factor or factors apparently distinct from any of the known nutrients which promoted a significant increment in body weight in immature guinea pigs fed a mineralized spray-process dried-milk ration under conditions of the present experiment.[11]

In addition to alfalfa, dehydrated rye grass, orchard grass, wheat grass, fescue grass, oat grass and tuna solubles were also found to promote a significant increment in body weight under conditions of the present experiment. Supplements of fresh lettuce, however, were without growth-promoting effect. In the latter respect the findings differ from those reported by Kohler et al. ('38) who found lettuce a potent source of "grass juice factor" activity. This finding suggests that the active factor(s) in the present experiment may be distinct from the "grass juice factor" of Kohler et al. ('38).

In contrast to the results obtained with a mineralized dried-milk ration, supplements of alfalfa meal did not promote a significant increment in body weight in guinea pigs fed a

[11] The growth-promoting factor in alfalfa and other succulent plants has tentatively been designated the M.R. (milk ration growth-promoting) factor. One unit has arbitrarily been defined as the minimum amount of material which when fed daily for 28 days to immature guinea pigs on a basal mineralized dried milk ration will promote an average weight increment of 2 gm per day over that of guinea pigs fed the basal ration alone. One unit of M.R. factor activity is exhibited by approximately 1 gm of whole alfalfa meal.

highly purified semi-synthetic diet. These findings suggest that the nutritional requirements of guinea pigs fed a mineralized dried-milk ration might be different from those of animals fed the highly purified diet. The growth retardation, unthrifty appearance, ruffled fur, varying degrees of alopecia and the bloated "pot-bellied" appearance of guinea pigs fed the unsupplemented mineralized dried-milk ration are symptoms comparable to those observed in other species of animals following the ingestion of diets with a high lactose content. The relative absence of these symptoms in animals receiving a similar diet supplemented with alfalfa and other succulent plants suggested the possibility that these materials contained a factor(s) that enabled the guinea pig to utilize lactose-containing rations more effectively. No deleterious effects were observed, however, when guinea pigs were fed a purified diet with a lactose content comparable to that of the unsupplemented mineralized dried-milk ration. It is possible that the lactose as present in a dried-milk ration may be utilized less efficiently by the guinea pig or may promote a different type of intestinal flora than a similar amount of lactose in a semi-synthetic diet.

The diverse effects of alfalfa meal supplementation between guinea pigs fed a mineralized dried-milk ration and those on a purified diet might also be due to the possible presence of toxic substances in the spray-process dried-milk powder. Data are available indicating that the heat-processing, drying and storage of milk results in deterioration of its nutritive value and other deleterious effects (McCollum and Davis, '15; Fairbanks and Mitchell, '35; Griswold, '51; Kraft and Morgan, '51). The relations of lactose and protein are particularly significant in respect to the nutritive changes which may occur in dry-milk products (Patton and Flipse, '53). It has been demonstrated that alfalfa meal contains a factor(s) apparently distinct from any of the known nutrients that counteracted the effects of toxic doses of iodinated casein (Tappan et al., '53), glucoascorbic acid (Ershoff, '54) and alpha-es-

tradiol (Ershoff et al., '56). It is possible that the beneficial effects of alfalfa (and other succulent plants) on guinea pigs fed a mineralized spray-processed dried-milk ration were due to the presence of a similar "antitoxic factor" (or factors) in these materials.

SUMMARY

Supplements of desiccated whole alfalfa promoted a significant increment in body weight in immature guinea pigs fed a mineralized spray-process dried-milk ration. Both the dried alfalfa juice and the water-washed pulp remaining after the extraction of the juice exhibited growth-promoting activity. The protective factor(s) in alfalfa is apparently distinct from any of the known nutrients. Supplements of dehydrated rye grass, orchard grass, wheat grass, fescue grass, oat grass and tuna solubles also showed significant growth-promoting activity. In contrast to the results obtained with a mineralized dried-milk ration, supplements of alfalfa meal did not promote a significant weight increment in immature guinea pigs fed a semi-synthetic diet.

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